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DEVELOPMENTAL STUDIES
AND
LABORATORY INVESTIGATIONS
Conducted by
Veterinary Services Diagnostic Laboratories
Fiscal Year 1972

Animal and Plant Health Inspection Service
U.S. DEPARTMENT OF AGRICULTURE

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DEVELOPMENTAL STUDIES AND LABORATORY INVESTIGATIONS CONDUCTED BY VETERINARY SERVICES DIAGNOSTIC LABORATORIES FISCAL YEAR 1972

ANAPLASMOSIS

PRODUCTION OF ANAPLASMOSIS CARD TEST ANTIGEN. Martin, W. H. and Ritchie, W. H. (Project Report)

Summary

By means of rapid serial passage of *Anaplasma marginale* in splenectomized calves maximum parasitemia was obtained which, when passed into grown whole animals, yielded sufficient card test antigen for approximately 225,000 tests. Part of this antigen is to be used for the 1973 fall test season and the remainder to be converted to a complement fixing antigen suitable for microtiter use.

Introduction

Since 1949, the complement fixation test for anaplasmosis has been the only reliable laboratory diagnostic tool available. In 1962 Ristic[1] suggested the micro capillary agglutination test as a method by which the veterinarian, himself, could diagnosis the disease in his own facility. However, its reliability has been questioned by many investigators and its manufacture has since been discontinued. Today, the anaplasmosis buffered antigen (ABA) card agglutination test appears to be an excellent supplement to the cumbersome and complex complement fixation (CF) test. Its accuracy approaches that of the CF test and in some instances surpasses the diagnostic accuracy during the early stages of the disease. Initially, it could only be used as a plasma test and was accurate only if the sample was a few hours old. Amerault[2] showed the addition of normal bovine serum to the antigen at the time of the test would allow the testing of serum several days old.

Materials and Methods

The strain of *Anaplasma marginale* used in production of card test antigen is identified as Virginia number 4. It was received from Veterinary Science Research in a state of high reproduction and high parasitemia in the host. Hopefully the organism would double the number of cells infected each day after inoculation. This particular strain of the organism was used in the large scale CF antigen production at Texas A&M in 1962-1963,[3] and in prior lots of ABA prepared for trial use. Although other strains do yield satisfactory antigens, this has been most satisfactory because higher parasite counts are obtained in a much shorter period of time. A rapidly reproducing organism appears to reach higher parasite counts in a shorter period of time with only slight drop in hematocrit (PCV).

Antigen production methods follow one described by Amerault and Roby[4] in which highly parasitized cells are ruptured by means of the French Pressure cell (FPC). Released "anaplasmata" are purified by repeated washings in sterile antibiotic saline. Amerault and Roby[5] suggest a soluble *A. marginale* antigen does exist and perhaps does play a part in the overall fixation of complement in the presence of positive serum, but it represents a small fraction of the total red blood cell (RBC) protein and can only be detected by a macroagar diffusion test. This antigenic fraction of the *A. marginale* organism is therefore lost during the washing process.

The animals used for both production runs of the antigen were aged female Holsteins purchased locally, and were negative for anaplasmosis by the CF and card tests. By rapid passage through splenectomized calves, a maximum parasite count was obtained in the shortest period of time. Inoculums consisted of cells collected in Alsevers solution. These cells were washed three times with cold sterile saline and inoculated at a rate of 1.0 ml packed cells per pound of body weight of the recipient animal. Production animals were not splenectomized. These animals were bled each morning and temperatures were taken twice daily. As the critical parasite—PCV levels were approaching, animals were bled both in early morning and mid-afternoon. The highest maximum counts were 70 percent, reaching these levels in 5 to 7 days. Table 1 shows the incubation period and hematological data for the first production run.

Although a 70 percent parasitemia with many multiple organisms per cell is average, it was not as high as the 80 to 90 percent parasitemias in some earlier productions. Both cows 9346 and 9348 yielded antigens of high potency, but the total volume of finished product from each animal was not high.

A second production run began again with blood from the carrier animals, this time passing through three splenectomized calves and then to production cows. These calves weighed 300 to 700 pounds. It was necessary to use all cells from the infected calves for passage to the next animal leaving none for production purposes. This is shown in table 2.

[1] Restie, M. A capillary tube-agglutination test for anaplasmosis — a preliminary report. J.A.V.M.A. *141*, 588-594 1962.

[2] Ameralt, T. E. A modified card agglutination test for bovine anaplasmosis; evaluation with serum and plasma from experimental and natural cases of anaplasmosis. 76th Annual Meeting U.S.A.H.A. November 1972.

[3] Franklin, T. E., Heck, F. C. and Huff, J. W. Anaplasmosis complement fixation antigen production. J.A.V.M.A. *24*, 483-487 1963.

[4] Ameralt, T. E. and Roby, T. O. A rapid card agglutination test for bovine anaplasmosis. J.A.V.M.A. *153* 1828-1834 1968.

[5] Ameralt, T. E. and Roby, T. O. Preparation and characterization of a soluble *anaplasma marginale* antigen. Vet. Res. *28* 1067-1072 1967.

Table 1.—Summary of production data from first production run

Animal identity	Aged carrier 5447	Calf 6010	Holstein cow 9346	Holstein cow 9348
Animal weight-pounds	—	— —	1400	1480
Percent parasite count at bleeding	0	63	70	70
Percent Hematocrit	32	16	24	19
Incubation period, days	— —	25	6	7
Wet weight A. marginale organisms-grams	— —	— —	20.4	27.9

Table 2.—Summary of production data from second production run

Animal identity	Aged carrier 5988	Calf 494	Calf 491	Calf 21	Holstein cow 982	Holstein cow No tag	Holstein cow 5137	Angus cross cow 22
Animal weight, pounds		325	350	700	1500	700	1200	800
Maximum parasite count-percent	0	68	72	80	77	70	62	77
Percent Hematocrit	34	20	15	25	22	19	18.5	25
Incubation period, days	— —	14	5	5	5	5	5	6
Wet weight A. marginale organisms grams	— —	— —	— —	— —	52.9	— —	23.1	19.7

Splenectomized calf 949 took 14 days to produce the highest parasitemia. However, cells from this animal injected into splenectomized calf 491 took only 5 days to reach an even higher level of cells parasitized. Passage of cells from 491 to splenectomized calf 21 again took only 5 days with maximum parasite count now at 80 percent. From this point, only whole production animals were used. Parasitemia remained above 70 percent except for cow 5137 on all subsequent inoculations. Cow 982, which had a relatively high parasite count (77 percent) and weighed the most, yielded the largest wet weight of A. marginale cells.

Results

The yield in total test doses and the number of test doses per ml of packed cells processed from each animal is shown in Table 3. The first four animals processed were approximately equal in the number of test doses per ml of packed cells processed; however, animal number 22 shows almost a 33 percent increase (14.2) over the average of the first four animals. This may be due to several factors associated with the animal itself. This animal had been splenectomized as a calf approximately 1 year ago and used as a test animal for subinoculation from a

Table 3.—Summary of data from two production lots of Anaplasmosis antigen

Animal number	9346	9348	982	5137	22
Percent Parasitemia	70	70	77	62	77
ml packed cells processed	2,800	3,800	6,750	3,250	2,000
Antigen yield—test doses	29,580	40,455	76,705	33,495	28,565
Test doses/ml packed cells	10.5	10.6	11.4	10.3	14.2

complement-fixiation-suspicious reaction card, negative reaction test animal. The fact that the PVC was almost normal (25 percent) also makes more cells available for processing. We do not know if any of these factors played a quantitative role in the antigen production. The use of cows which have been splenectomized as calves should be investigated in the future.

Conclusion

We have produced a card agglutination antigen from five animals yielding slightly more than 200,000 test doses at ABA. However, not all this is to be used for this specific product. In its unstained bulk form it serves as an excellent quality, highly antigenic complement fixing antigen. The present CF antigen is old (1962) and contains many impurities such as hemoglobin, cell stroma particulate forms, and other cellular debris. With the ABA all these are removed and its reactions to test serums are identical to the old antigen.

It is significant that with the massive inoculations made into these animals, with the resultant short incubation period, the majority of animals do not develop any detectable complement fixing or agglutinating antibodies. In those cases where significant CF titers develop, that particular antigen is usually unsatisfactory.

BOVINE VIRAL DIARRHEA

Sixty-six pigs were exposed experimentally to bovine viral diarrhea (BVD) virus. Several strains of the virus and routes of administration were utilized. The immunologic, pathologic, serologic, and virologic responses of pigs in selected groups were determined.

In a group of pigs exposed twice to a field strain of virus, the serologic responses consisted of moderate to high BVD antibody titers and low cross-neutralizing titers against hog cholera (HC) virus. When given virulent HC virus, severe clinical reactions occurred in the BVD-exposed pigs, but eight of nine pigs survived.

Further evidence of a viremia was established by the isolation of BVD virus from seven pigs. Isolates were cultured from the lungs of one pig and the blood of five pigs exposed intranasally (I.N.) In two pigs, the blood isolates were detected 14 days postexposure (DPE). Isolates were recovered from the ileum, mesenteric lymph nodes, and spleen of one pig exposed intramuscularly (I.M.).

In two swine herds investigated for HC, serologic tests for BVD were done after low to moderate HC antibody titers were detected. In each case, higher BVD antibody titers were detected, suggesting that the former were cross-neutralization titers resulting from BVD infection. One herd had been in contact with BVD-vaccinated cattle and the other herd had been fed bovine offal.

BRUCELLOSIS

EVALUATION OF FIVE MEDIUMS FOR THE STABILIZATION OF <u>BRUCELLA ABORTUS</u> STRAIN 19 DESICCATED BY LYOPHILIZATION. Angus, R. D., Love, E. L., and Pietz, D. E.
(Project Report)

Summary

Lyophilized *Brucella abortus* Strain 19 vaccines were prepared using five recommended stabilizing mediums. These five vaccines were compared with each other and with liquid vaccine relative to their suitability for (1) Maintaining distinguishing cultural characteristics; (2) maintaining viability during lyophilization; (3) quality of restoration; (4) maintaining viability under various storage temperatures; and (5) maintaining stable, antigenic and virulence qualities.

Introduction

Brucella abortus Strain 19 has been widely used for many years as a vaccine to increase the resistance of cattle to naturally occuring brucellosis. This strain of *Brucella* was originally isolated by personnel of the USDA in 1923 and the foundation culture is now maintained by the Biologic Reagents Section, Veterinary Services Diagnostic Laboratory (VSDL), APHIS, Ames, Iowa.

Seed cultures have been distributed worldwide for production and reference purposes and the World Health Organization recently designated the Biologic Reagents Section, VSDL, as the source for "Original Seed". Since "Original Seed" was to be supplied in the lyophilized form, a stabilizing medium that would best preserve the characteristics of Strain 19 with maximum viability was desirable. Several mediums have been used and recommended, (1,2,5,6,7)[1] but these have not been examined on a direct comparative basis.

This project was designed to determine which of these five mediums would be the most suitable stabilizer for *B. arbortus* Strain 19 vaccine and "Original Seed".

Materials

A. Culture: *Brucella abortus* Strain 19, USDA Stock, serial 72, (September 1, 1962), was selected because this serial had typical characteristics and was used as the control for the characterization of *Brucella abortus* Strain 19 (4).

B. Mediums:
1. One percent peptone (2) was used in making vaccine dilutions for conducting viability counts.
2. Tryptose agar (3) was used for performing viability counts.
3. Potato-infusion agar (2) was used for culture selections, vaccine production, and colonial index evaluations.

C. Stabilizing Mediums:
1. Saline-Skim Milk. (S-M). This medium has been used routinely by the USDA for lyophilizing *Brucella* seed cultures. The *Brucella* are washed from the medium with phosphate-buffered saline, pH 6.4, (P.B.S.) to which is added an equal volume of skim milk that was sterilized by autoclaving (2).
2. Modified Naylor-Smith. (N-S). This medium was used in studies on the lyophilization of Brucella and consists of a solution of proteins, carbohydrates, and various salts (7).
3. Lactose-Salt. (L-S). This medium was originally recommended for a variety of organisms, including *Brucella*, and contains lactose and organic and inorganic salts (6).

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[1] Numbers in parentheses refer to References at end of this paper.

4. World Health Organization. (WHO). This medium was recommended by experts publishing under the auspices of the World Health Organization and consists of a solution of protein, sucrose, and organic acid (1).

5. Zoonosis Center. (Z-C). This medium has been used for vaccine preparation by the Zoonosis Center, Pan American Health Organization, Buenos Aires, Argentina (5), and consists of the following:

[2]Bacto casitone	50 gm
Sodium glutamate	20 gm
Bovine albumin	20 gm
[3]Polivinal pirolidon	10 gm
Distilled H_2O q.s.	1000 ml

D. Lyophilization Apparatus: A RePP Model 41, manufactured by the Virtus Company, Gardiner, New York, was used for lyophilization.

E. Vials and Closures: The vials, which were made of boro-silica glass and were 10 ml in size with a 13 mm opening, were closed with silicone treated, split, butyl rubber stoppers and sealed with aluminum caps.

Methods

A. Vaccine Preparation and Lyophilization. A *B. abortus* Strain 19 seed culture was selected and prepared in P.B.S. This preparation was used as the inoculum for Roux bottles of potato-infusion agar. Incubation was for 48 hours at 37° C. The cells were harvested by gently washing the agar with 30-35 ml of P.B.S. The cell suspensions from about 10 Roux flasks were pooled in one flask. Sterility tests were conducted on each pool. The final cell suspension was prepared by combining 10 pools representing 104 Roux flasks. Sterility, viability, and colonial morphology tests were conducted on the final suspension. Preliminary studies indicated a probable lyophilization loss of about 50 percent and a desired vial fill volume of 3 ml. Based on these data the final cell suspension was diluted at a ratio of 335 ml to 1,000 ml of each of the suspending mediums. Each of the vials were filled with a 3 ml volume and immediately frozen on the lyophilizer shelves to a temperature of -50 to -60° C. Prior investigation had revealed that neither the shelf, nor the location on a shelf, had any perceivable effect on the resulting viability. The lyophilization process was completed in 24 hours at which time the temperature of the vials was 28° to 30°C. The vials were sealed while at the original vacuum of under $50\,\mu$. The standard liquid control (L-C) vaccine was prepared from the final cell suspension by diluting with P.B.S.

B. Vaccine Storage. The vials of each lyophilized vaccine were arbitrarily divided into five groups and stored in the dark at temperatures of 37° C., 25° C., 4° C., -25° C. and -50° C. The liquid control vaccine was also divided into five groups and stored at the same temperatures.

C. Vaccine Evaluation. The post-lyophilization evaluation was conducted on six arbitrarily selected vials of each vaccine. After storage, the evaluations were based on the results obtained from three arbitrarily selected vials of each vaccine at each storage temperature. All evaluations for a given time and storage temperature were conducted at the same time. Lyophilized vaccines were reconstituted with 5 ml of double distilled water and examined for quality of restoration. Cultures from each of the selected vials were examined for colonial morphology, viability, dissociation and purity. The pH value was determined on a pool of the selected vials of each vaccine at each time and storage temperature. All viability counts were made at 10^{-9} dilution and the viability was considered zero when there was no growth of colonies from the three selected vials.

Each vaccine was tested for antigenicity and virulence in 12 guinea pigs as previously described (8).

A culture from each lot of vaccine was characterized by conventional and definitive typing procedures (1,4).

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[2]Same as enzymatic digest of casein.
[3]Poly Vinyl Pyrrolidone (Plasdone C).

Results

After combining the final cell suspension with the Z-C medium, and during the dispensing operations, it was observed that the cells tended to settle out of suspension. This condition was not observed in the vaccines prepared with the other mediums. The pre- and postlyophilization viability counts and the percent survival after lyophilization for each vaccine are recorded in table 1.

Table 1.—Pre- and post-lyophilization viability counts[1]

Medium	Prelyophilization	Postlyophilization	Percent Survival
S-M	25.200	12.650	50.20
N-S	28.800	12.166	42.24
L-S	28.350	20.200	71.25
WHO	29.550	14.425	48.82
Z-C	28.150	14.008	49.82
L-C	15.708	—	—

[1] $\times 10^9$/ml.

All the vaccines rehydrated fairly well. The N-S, L-S and WHO mediums dissolved in under 30 seconds. The S-M medium dissolved in less than 60 seconds, but always contained a white residue. The residue resembled the "curd" normally formed by milk in the presence of acid. The residue could be broken up fairly well by vigorous agitation but remained as a fine suspension rather than going into solution. The Z-C medium dissolved more slowly, requiring 1 to 4 minutes, and occasionally up to 10 minutes, to be completely dissolved. All restored vaccines appeared homogenous except for the "curd" in the S-M. All vials of vaccine were found to be sealed and maintaining vacuum at the time evaluated. The colonial morphology of each vaccine was typical of *B. abortus* Strain 19. There was no evidence of dissociation in each of the vaccines and there was no contamination detected.

The conventional and definitive typing results on cultures of each vaccine were within the normal expected ranges and patterns for *B. abortus* Strain 19 and were comparable to the results obtained on the prelyophilization stock culture (4).

Each vaccine, when injected into 12 guinea pigs, produced an antigenic response and infection level that was comparable to what has been previously reported for Strain 19 (9).

The results on the maintenance of viability of each vaccine stored at $37°$ C., $25°$ C., $4°$ C., $-25°$ C. and $-50°$ C. are recorded in tables 2, 3, 4, 5 and 6 respectively. When stored under refrigeration, which is recommended, the most satisfactory results were obtained with N-S, L-S and WHO mediums (table 4). The viability of L-C was satisfactory after 92 days at $4°$ C., but not after 182 days.

At room temperature ($25°$ C.) the most satisfactory results were obtained with the WHO medium (table 3). The results obtained with S-M, L-S and Z-C mediums were comparable to each other.

The most satisfactory results at the storage temperature of $37°$ C. were obtained with S-M, WHO and Z-C mediums (table 2).

The viability counts on the vaccines stored at $-25°$ C. were satisfactory for all vaccines except L-C; however, the most satisfactory results were with the WHO medium as a 100 percent viability was maintained for the 470-day period (table 5).

At $-50°$ C., the viable counts for all vaccines were satisfactory, but the counts on the WHO vaccine were more stable than the counts of the other vaccines (table 6).

Table 2. Maintenance of viability of each vaccine stored at 37° C.

Storage time (days)	Percent survival					
	S-M	N-S	L-S	WHO	Z-C	L-C
0	100	100	100	100	100	100
7	39	2	69	73	70	62
14	45	<1	41	70	70	6
21	38	<1	33	53	53	0
27	35	0	20	48	47	
35	28		14	37	49	
42	28		9	26	46	
49	27		5	21	31	
56	30		5	17	36	
62	32		7	22	36	
71	30		6	27	23	
84	29		2	15	21	
98	24		4	15	12	
112	22		5	10	13	
130	15		<1	4	9	
148	19		2	5	3	
175	7		<1	2	4	
203	5		<1	1	2	
237	5		<1	<1	<1	
265	6		0	<1	0	
295	0			0		

Table 3.—Maintenance of viability of each vaccine stored at 25° C.

Storage time (days)	Percent survival					
	S-M	N-S	L-S	WHO	Z-C	L-C
0	100	100	100	100	100	100
32	53	63	68	90	84	57
60	45	67	77	92	56	11
90	38	51	65	100	49	8
119	42	26	50	100	46	1
152	27	11	45	79	29	<1
180	28	1	32	79	21	<1
210	34	7	36	66	24	0
238	35	7	31	71	28	
266	30	4	33	68	17	
309	28	4	28	59	28	
330	28	<1	21	57	15	
361[1]						
462	18	7	13	48	9	

[1] Data in error due to laboratory problem.

Table 4.—Maintenance of viability of each vaccine stored at 4° C.

Storage time (days)	Percent survival					
	S-M	N-S	L-S	WHO	Z-C	L-C
0	100	100	100	100	100	100
92	73	100	100	100	73	81
182	32	100	79	100	71	21
270	48	82	92	92	46	5
361[1]						
397	28	26	53	72	18	0
463	28	73	66	93	36	

[1] Data in error due to laboratory problem.

Table 5.—Maintenance of viability of each vaccine stored at $-25°$ C.

Storage time (days)	Percent survival					
	S-M	N-S	L-S	WHO	Z-C	L-C
0	100	100	100	100	100	100
92	100	79	100	100	100	3
179	100	32	95	100	100	1
267	79	83	96	100	74	0
360[1]						
406	53	41	91	100	75	
470	74	55	82	100	68	

[1] Data in error due to laboratory problem.

Table 6.—Maintenance of viability of each vaccine stored at $-50°$ C.

Storage time (days)	Percent survival					
	S-M	N-S	L-S	WHO	Z-C	L-C
0	100	100	100	100	100	100
92	100	68	97	100	100	46
182	100	100	88	83	97	37
268	100	100	94	96	84	48
365[1]						
406	82	42	82	98	67	20
471	100	94	89	97	100	28

[1] Data in error due to laboratory problem.

Discussion

The five mediums were relatively equal in suitability for maintaining the colonial and typing characteristics, and the antigenic and virulent qualities of *B. abortus* Strain 19. There was some variation between the pH of the vaccines prepared with the various mediums; however, the pH of each vaccine remained relatively stable during storage. Therefore, restoration quality and maintenence of viability were used as the criteria for selecting the most satisfactory medium.

The Z-C and S-M mediums were eliminated on their quality of restoration characteristics. The Z-C medium tended to settle out, both in the prelyophilized state and in the reconstituted state. The S-M medium did not restore readily, requiring vigorous agitation for resuspending and even then fine particles remained in suspension.

The percent survival of *Brucella* following lyophilization was similar for each medium except for L-S, which had a relatively higher survival rate (table 1). In this aspect the L-S was significantly superior to the other stabilizers.

Since it is recommended that lyophilized cultures and vaccines be stored at $4°$ C., but may sometimes be stored at room temperature ($25°$ C.) and occasionally be subjected to higher temperatures ($37°$ C.), the maintenance of viability at these three storage temperatures was the major factor in evaluating the remaining three (L-S, N-S and WHO). A graphic comparison of the viability of the vaccines prepared with these three mediums and stored at these three temperatures is presented in figures 1, 2 and 3.

The percent of viable *Brucella* was highest in the vaccine prepared with the WHO medium at all three storage temperatures and the percent of viable *Brucella* was the lowest in the vaccine prepared with the N-S medium. Therefore, when all parameters of evaluation were considered, the vaccine prepared with the WHO medium was superior and is now being utilized for producing *B. abortus* Strain 19 vaccines and "Original Seed".

Since the loss of viability from the lyophilization process was considerably less in the vaccine prepared with L-S medium, it may be possible to prepare a more satisfactory vaccine with a medium prepared from components of the L-S and WHO mediums. This may be worthy of investigation at a later date.

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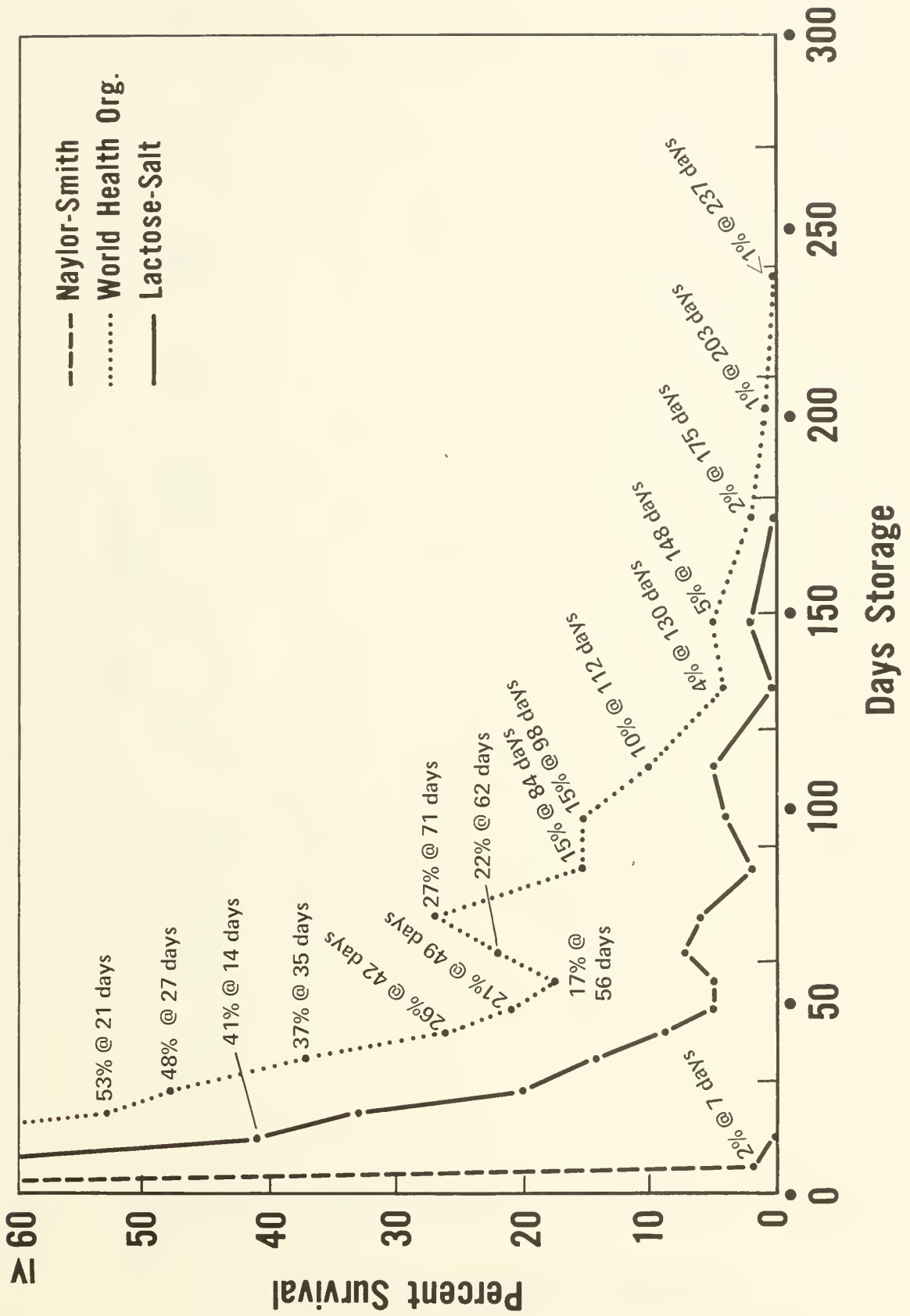
Figure 1.—Maintenance of viability of vaccine stored at 37° C.
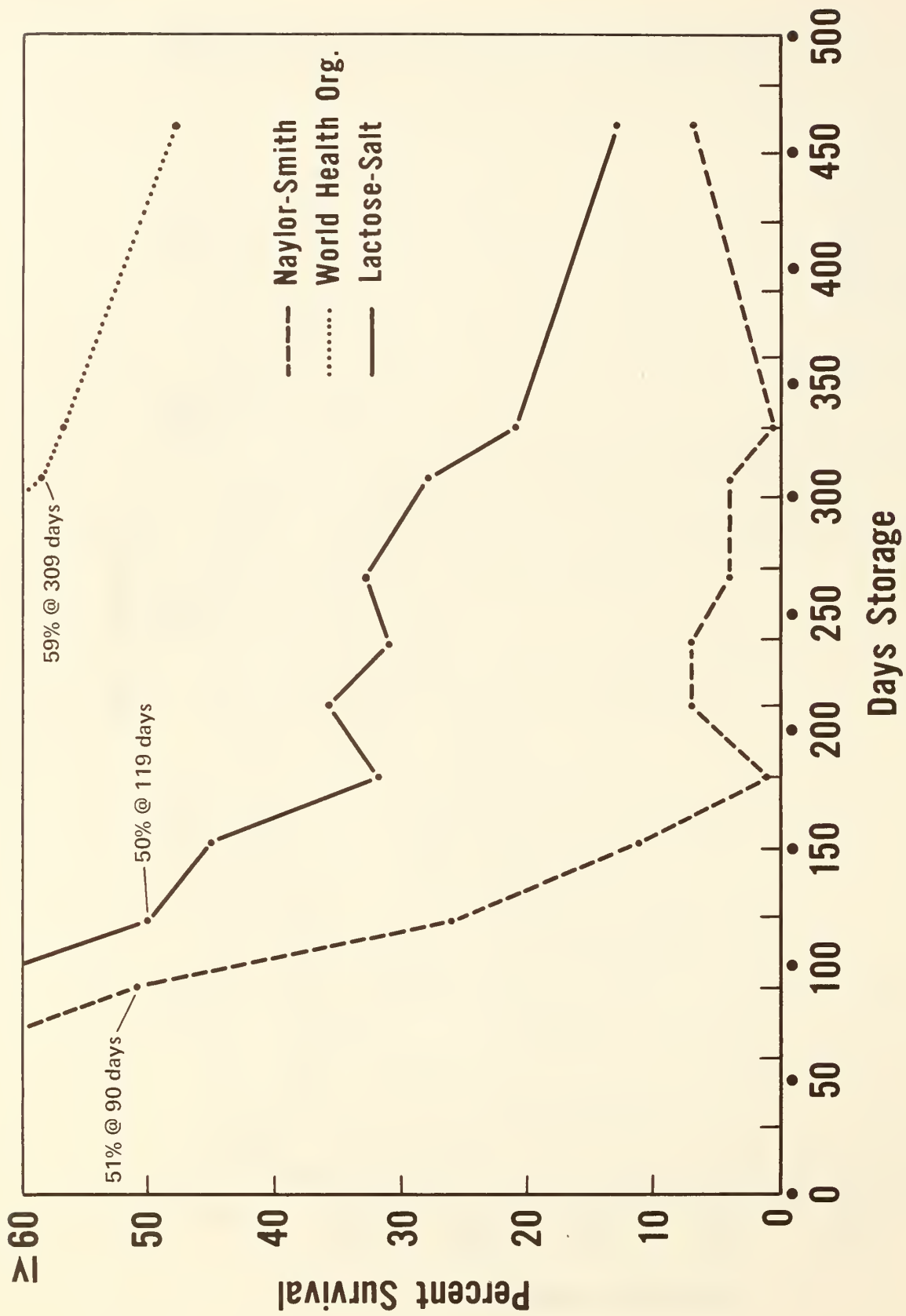
Figure 2.—Maintenance of viability of vaccine stored at 25° C.

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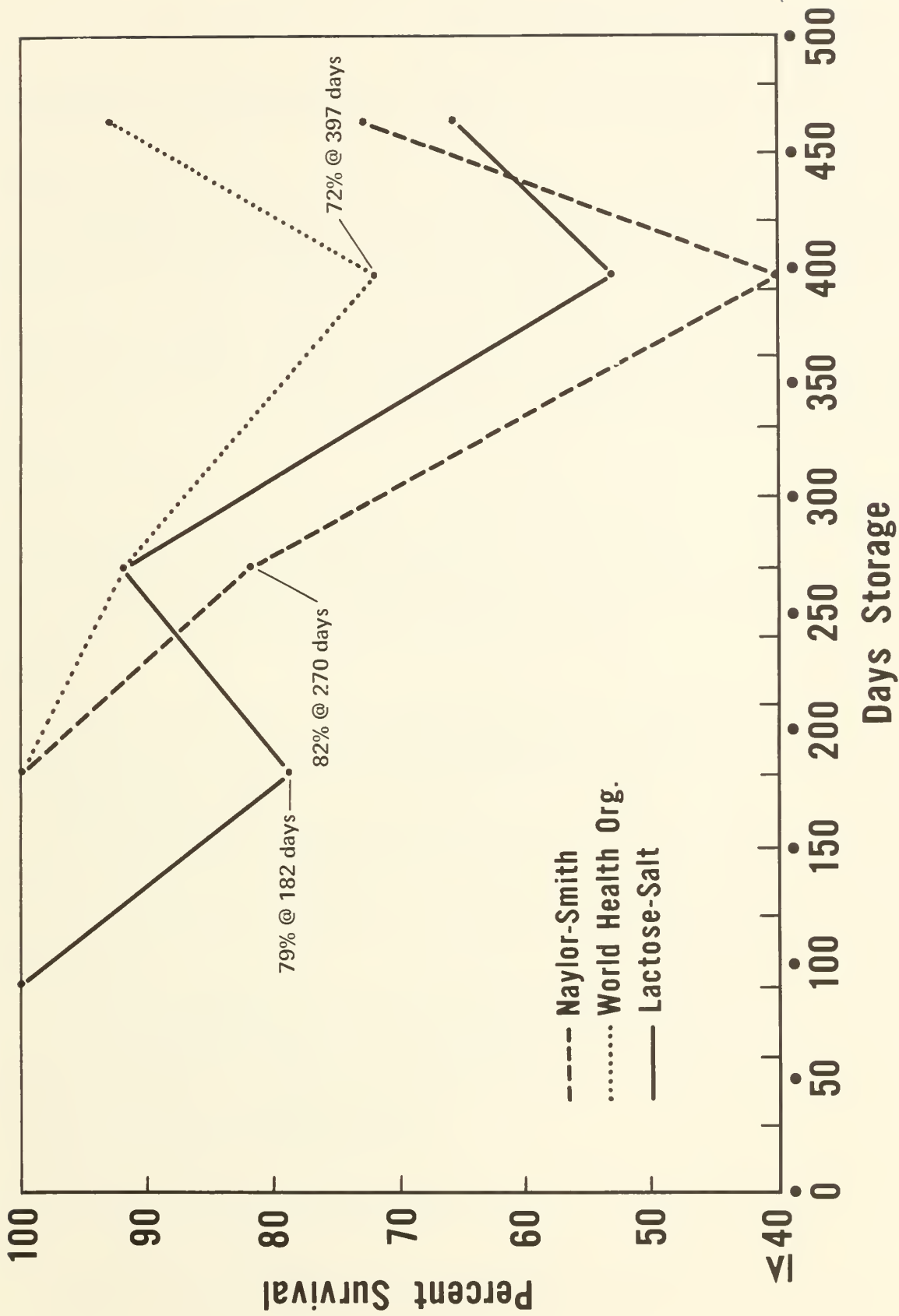
Figure 3. —Maintenance of viability of vaccine stored at 1° C.

Columbia, Targhee and Rambouillet rams were artificially exposed to *Brucella ovis* via the conjunctival sac. Rams not shedding *B. ovis* in their semen were re-exposed intravenously 13 weeks, and again 27 weeks, after the initial exposure.

Ewes, induced to come in estrus, were mated with rams that were shedding *B. ovis* in their semen. Subsequently, during the same period of estrus, each ewe was mated with two noninfected vaccinated or nonvaccinated rams. After mating, the infected rams were placed in a pen with vaccinated and nonvaccinated rams. These rams were allowed to cohabit for approximately 1 year.

Serologic responses occurred in all 48 artificially exposed rams, in 8 of 25 venereal-contact, non-vaccinated rams and in 5 of 23 cohabitation-contact nonvaccinated rams. The serologic responses of the venereal and cohabitation contact, vaccinated rams were difficult to evaluate because of persistent vaccinal titers.

Brucella ovis was isolated from the semen of 14 of 28 artificially exposed rams. Single colony isolations were made from the semen of each of two cohabitation contact, vaccinated rams on one occasion. There was no other cultural evidence of transmission of infection by venereal or cohabitation contact.

HOG CHOLERA

EVALUATION OF A PORTABLE DRY ICE MACHINE. Ellis, E. M. (Project Report)

Summary

A dry ice making machine was tested in two hog cholera field stations. Although usable dry ice was produced, erratic results were obtained, indicating that operator technique was a large factor. In spite of these deficiencies, use of the machine was recommended by field personnel in areas where no dry ice was available.

Introduction

A commercial dry ice making machine, which was very portable, was tested for its usefulness in the hog cholera eradication program to supply dry ice for preserving hog cholera tissues enroute to the laboratory. The machine was tested at the Stephenville, Tex., hog cholera field office and at the Brownwood hog cholera field office.

Materials and Methods

A Chemical Rubber Company[1] "Frigimat" dry ice maker was tested. The machine consisted of a pressure chamber in which the CO_2 gas was compressed into ice, an inlet valve, and a relief valve.

The machine was assembled according to the directions supplied. A length of steel tubing connected the inlet valve to a tank of bone dry liquid CO_2 gas. CO_2 gas was allowed to flow into the chamber for varying periods of time. The chamber was then disassembled and the cylinder of ice removed.

Results

1. Results of use of the dry ice machine in the Brownwood hog cholera field office.

The dry ice machine was used in the Brownwood hog cholera field office for a period of approximately 2 weeks. A dry grade of CO_2 was obtained from a welding supply at the following cost:

20-pound cylinder - $6.00
50-pound cylinder - $11.00

The 20-pound cylinder was easier to use, but did not give satisfactory results. The first cylinder was practically lost due to error. The second 20-pound cylinder produced some usable dry ice but the quality was difficult to control.

The 50-pound cylinder gave overall good results. The ice was hard throughout and two blocks appeared sufficient for one shipment. The equivalent of three shipments was obtained from the 50-pound CO_2 cylinder (see table 1, page 18).

2. Results of use of the dry ice machine in the Stephenville hog cholera field office.

Use of the portable dry ice machine at the Stephenville hog cholera field office presented several problems.

The first cylinder of *bone dry* grade of CO_2 produced only two satisfactory blocks of dry ice. This tank was probably only partially filled. Results thereafter were erratic, ranging from excellent to nothing and usually poor. At the present time, these results appear to have been due first to errors by the machine operator and later by the wrong grade of CO_2 being delivered by the supplier.

The proper grade of CO_2 was obtained in the last two bottles used in Stephenville and the results were satisfactory.

Use of the machine was favorably recommended by Dr. Herbert Tally, diagnostician in charge at the Stephenville office during the month of June (see table 2, page 18).

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[1] Chemical Rubber Company, 18901 Cranwood Parkway, Cleveland, Ohio 44128.

Table 1.—Results of use of the dry ice making machine at the Brownwood hog cholera field office

Machine capacity (pounds)	Quality	Date	Minutes in operation	Weight in block (pounds)	Ambient temperature (° F.)
20	Poor	3/31/71	3	3/4	80
	Fair	4/1/71	3	1	73
	Poor	4/2/71	1	1	76
	Good	4/2/71	1	1/3	76
	½ Powdery	4/2/71	4 3/4	4/5	77
50	Good	4/7/71	4	1 1/2	72
	Very good	4/7/71	3 5/6	1 3/4	72
	Fair	4/14/71	4	3/4	70
	Very good	4/14/71	4 1/2	1 3/4	75
	Do	4/15/71	4	1 3/4	73
	Good	4/15/71	3	1	73 not long enough

Table 2.—Results of use of the dry ice making machine at the Stephenville hog cholera field office

Machine capacity (pounds)	Quality	Date	Minutes in operation	Weight in block (pounds)	Ambient temperature (° F.)
50	Excellent	5/19/71	5 1/4	1 3/4	76
	Good, full size but soft	5/19/71	3 1/2	1 1/4	76
	Nothing	5/21/71	7	0	85
	Do	5/21/71	6 1/2	0	85
	Do	5/24/71	6	0	76
	Do	5/24/71	6	0	76
50	Snow-full	5/24/71	5	1 1/2	76
	Hard snow	5/24/71	4	1 3/4	76
	Do	5/24/71	5	1 3/4	76
	1/3 of cyl.	5/24/71	5	1/2	76
	None	5/24/71	5	0	76
	Do	5/24/71	5	0	76
	Nothing	Tried gas tank upright			
	Do	Layed tank down again			
		Tank valve down, bottom of tank elevated			
	Snow	5/25/71	5	1/2	76
	Hard snow	5/25/71	5	1 3/4	76
	Do	5/25/71	5	3/4	76
	Empty	5/25/71	5	0	76
50	0	6/1/71	5	0	76
	Snow type	6/1/71	5	1/4	76
	Do	6/1/71	5	1	76
	Hard snow	6/1/71	4 1/2	1	76
	Do	6/6/71	5	1/4	74
	Hard snow	6/6/71	5	1/4	74
	Do	6/6/71	5	3/4	74
	Hard snow	6/6/71	5	1/2	74
	Med. hard snow	6/6/71	5	1/2	74
	Do	6/6/71	5	1/2	74

Machine capacity (pounds)	Quality	Date	Minutes in operation	Weight in block (pounds)	Ambient temperature (° F.)
50	Hard	6/7/71	5	3/4	76
	0	6/7/71	5	3/4	76
	0	6/8/71	4 1/2	0	76
	0	6/8/71	6	0	76
50	0	6/8/71	6	0	76
	Snow type	6/10/71	6	3/4	76
	Do	6/10/71	5	3/4	76
	Do	6/10/71	5	3/4	76
	Do	6/11/71	5	3/4	76
	Do	6/11/71	5	3/4	76
	0	6/14/71	5	0	78
	0	6/15/71	5	0	76
50	Good	6/16/71	5	1	76
	Med. Hard Snow ...	6/16/71	5	1	76
	Excellent	6/20/71	6	1 1/4	76
	Do	6/20/71	7	1 1/2	76
	Good Snow type ...	6/23/71	7	1	76
	Snow type	6/23/71	7	1/2	76
50	Good	6/23/71	7	1	78
	Do	6/23/71	7	1	78
	Excellent	6/26/71	7	1 1/2	76
	Do	6/26/71	7	1 1/2	76
	Do	6/26/71	7	1 1/2	76
	Do	6/28/71	7	1 1/2	76
	Good	6/28/71	6	1 1/2	76
50	Good	6/30/71	7	1 1/4	78
	Do	6/30/71	6	1 1/4	78
	Excellent	7/1/71	8	1 1/2	78
	Do	7/2/71	6	1 1/2	78

Discussion

The ice machine required some experience to obtain good results. Bone dry CO_2 was required in a tank having a siphon tube. Even though use of the machine was recommended, examination of the results appears to indicate that the equipment produced sporadic results. The price per pound averaged $1.30. It was recommended that the machine be turned over to Emergency Diseases and that an attempt be made to apply it to an emergency disease situation.

Even though erratic results were obtained, the machine was recommended for use by the hog cholera field stations involved.

FURTHER STUDIES ON INACTIVATION OF HOG CHOLERA VIRUS IN BRAIN TISSUE.

Miller, L. D., Jones, N. K., and Muhm, R. L. (Project Report)

Summary

Viable hog cholera virus was previously demonstrated in intact pig brains that had been immersed in 10 percent buffered formol saline for 24 hours.

In this study, two modified procedures were used for tissue fixation. Serial transverse cuts were made in brains prior to fixation for 24 hours in 10 percent formol saline for one procedure. For the other, brains were fixed in toto for 20 to 24 hours before cutting and replacing in fixative for 4 additional hours.

Infectious hog cholera virus was not detected in brains fixed by either method. The testing procedure was inoculation of susceptible swine with fixed-brain extracts.

Introduction

As the hog cholera eradication program progresses, safety in the diagnostic laboratories has become increasingly important. Previous studies have shown that hog cholera virus can survive and remain infectious in pig brains that have been immersed in 10 percent buffered formol saline for 24 hours.[1] Infectivity was destroyed when cryostat sections (10 microns in thickness) were immersed in absolute ethanol for one minute.

The purpose of this study was to determine if infectious hog cholera virus would persist in:

1. Pig brains in which transverse cuts were spaced at approximately 1-inch intervals prior to fixation for 24 hours in buffered formol saline.

2. Brains that were fixed in toto for 20 to 24 hours, then cut transversely at 1-inch intervals and replaced in fixative for an additional 4 hours.

Materials and Methods

Pigs.— Thirty second generation specific pathogen free pigs weighing 90 to 130 pounds each were obtained from Animal Supply, National Animal Disease Laboratory. All pigs were healthy at the time of inoculation.

Virus.—For inoculation a virulent strain of hog cholera virus (Ames strain) was used.

Experimental Procedure.— Six pigs (group A) were inoculated intramuscularly with 1.5 cc of swine blood containing hog cholera virus. All pigs developed typical signs of hog cholera and were killed 5 to 6 days post inoculation (DPI). The brains were removed intact. To confirm the presence of infective virus, approximately 2 grams of brain tissue were taken from the olfactory area of each pig, homogenized with sterile sand in 8 to 10 cc of sterile saline. The supernate from each was inoculated into one pig (group B).

The remaining portion of each brain was immersed individually in 32-oz. glass jars containing 10 percent buffered formol saline. After 20 to 24 hours the brains were removed, cut transversely at 1-inch intervals, and replaced in the fixative for an additional 4 hours. Upon removal, about 2 grams of tissue were obtained from the dense thalamus-hypothalamus area, homogenized with sterile sand in 8 to 10 cc of sterile saline. The supernatant fluid from each preparation was injected into one pig (group C).

The pigs in group B (inoculated with fresh brain material) all developed hog cholera and were the source for material used in part two of the study. Fresh brain homogenates were prepared as described and the supernate from each was inoculated into another pig (group D). The remainder of each brain was cut transversely into slices 1-inch in thickness and fixed in 10 percent buffered formol saline for 24 hours. Then portions similar to those described above were prepared and injected into six more pigs (group E).

Pigs that did not develop hog cholera after injection of brain supernate were challenged with Ames virus after an observation period of 14 to 21 days.

The behavior, temperature, and total leukocyte count of each of the pigs were noted throughout the study.

Preinoculation serum samples were obtained from all pigs and a second sample was taken from the pigs in groups C and E prior to challenge.

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[1] Muhm, R. L., Jones, N. K., McDaniel, H. A. Inactivation of hog cholera virus in brain tissue. Ann. Rep. An. Hlth. Diag. Lab. Ames. Iowa, Vet. Serv., USDA, 1971.

Portions of tonsil, spleen, and mesenteric lymph nodes were taken as necropsy and frozen. The tissues were subsequently examined by the fluorescent antibody tissue section technique (FATST) to confirm infection with hog cholera virus.[2]

Results

Those pigs inoculated with infected swine blood (group A) and with fresh brain material (groups B and D) became acutely ill and were necropsied 5 to 8 days postinoculation (DPI). All pigs developed elevated temperatures (up to $108.2°$ F) and leukopenias (as low as 2,000 per mm^3 after inoculation. Clinical signs observed were inactivity, anorexia, ocular discharge, constipation, erythema, hyperesthesea and piling on top of each other when lying down.

The pigs injected with formalized brain material (groups C and E) remained healthy during the 14- to 21-day observation period. Temperatures and total leukocyte counts taken during that time were within the normal range. After challenge with virulent hog cholera virus, the pigs developed elevated temperatures, leukopenias, and clinical signs similar to those described above. All 12 pigs were necropsied 5- to 6-DPI.

Infection with hog cholera virus was confirmed in all 30 pigs by the FATST.

Discussion

The results of this study provide useful information for the development of practical and safe laboratory procedures for handling hog cholera infected tissues. Since the transport of specimens to the laboratory often requires 1 day, this project demonstrates that processing could begin immediately or after a minimum delay (4 hours) with little risk of viable virus remaining in the formalized brain.

The purpose for obtaining the pre-challenge serums from pigs in groups C and E was to test for hog cholera antibodies in the event the animals were resistant to challenge. It was thought the formalized brain material might contain sufficient hog cholera antigen to confer immunity in the inoculated pigs.

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[2] Bedell, D., McDaniel, H. A., Clark, C. D., Gray, A. P., and Aikin, J. Recommended minimum standards for detecting hog cholera viral antigen by the fluorescent antibody tissue section technique. Rep. Proc. USLSA, 72nd Ann. Mtg., October, 1968.

MECHANICAL TRANSMISSION OF HOG CHOLERA VIRUS TO PIGS WITH A SLAP TATTOO
INSTRUMENT. Stewart, W. C., Kresse, J. I., Erickson, G. A., and Carbrey, E. A.
(Project Report)

Summary

Susceptible pigs in one of two trials contracted hog cholera (HC) when slapped with a tattoo instrument that had previously been used to tatto a HC-infected pig. With this evidence, it was concluded that the slap tattoo instrument should not be used to identify market swine returning to farms. However, the instrument was considered to have some application in the identification of swine destined for immediate slaughter where traceback to the farm of origin is necessary.

Introduction

A slap tattoo instrument was proposed for use in the identification of swine in market channels. Before this approval could be granted for feeder pigs and breeding swine, it was necessary to ascertain if infectious diseases were transmitted by the instrument. The specific purpose of this investigation was to determine if HC virus was transmitted from pig to pig by the tattoo instrument.

Materials and Methods

Slap Tattoo Instrument.—The instrument employed was the type proposed for use in markets and was supplied by the Swine Diseases Staff, Hyattsville, Maryland (fig. 1).

BN-40751

Figure 1.—Slap tattoo instrument used in the hog cholera transmission experiments.

Virus.—The highly virulent Ames strain of HC virus was used. Blood was taken from a pig acutely ill with HC, defibrinated, and stored at −80 C.

Pigs.—Second generation, naturally farrowed, specific pathogen free (SPF) pigs weighing 30 to 40 lbs were used.

Cell Cultures.—Virus isolation attempts were made in PK-15 cell cultures. The cultures were harvested thrice weekly and propagated in F15 Eagle's Medium[1] with two percent SPF swine serum.

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[1] Grand Island Biological Company, Grand Island, N. Y.

Experimental Procedure.—The Ames virus was given intramuscularly to a pig (donor), and 6 to 7 days later when the pig was acutely ill, it was slapped in the hip or shoulder region with the tattoo instrument. The instrument was immediately placed in a closed metal container and taken to another room where a HC susceptible pig (recipient) was similarly slapped (fig. 2).

Figure 2.—Tattoo on a pig that contracted hog cholera after being slapped with the instrument.

Two trials were performed with separate donor pigs being used in each trial. Blood specimens in heparin were taken each time a donor pig was slapped, and the virus concentrations, expressed as plaque forming units (PFU), were determined by the fluorescent antibody cell culture technique (FACCT) as previously described.[1] In the first trial the three recipient pigs were slapped in rapid succession after initially pressing the tattoo instrument to the ink pad and slapping the donor pig. In the second trial the procedure was slightly modified. The instrument was pressed to the ink pad and the donor pig was slapped again prior to slapping each of the three recipient pigs.

Hog cholera virus was isolated from the recipient pigs by the FACCT employing blood or 33-1/3 percent splenic suspensions. The immunity of the recipient pigs that remained healthy was subsequently challenged by giving them 1 ml of the Ames virus intramuscularly. Serums taken from the pigs prior to administration of the Ames virus were tested for HC antibodies by the fluorescent antibody serum neutralization test.[2]

[1] Carbrey, E. A., Stewart, W. C., Kresse, J. I., and Lee, L. R. 1965. Technical aspects of tissue culture fluorescent antibody technique. Proc. 69th Annual Meeting U. S. Livestock San. Assoc.: 487-500.
[2] Carbrey, E. A., Stewart, W. C., Kress, J. I., and Lee, L. R. 1969. Confirmation of hog cholera diagnosis by rapid serum neutralization technique. Jour. Amer. Vet. Med. Assoc. 155: 2201-2210.

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Assay of Tattoo Ink for Virucidal Properties - A mixture of equal parts of ink[3] and virus was maintained at room temperature for 15 minutes. Ten-fold dilutions of the mixture were prepared and viral PFU were determined by the FACCT. A replicate titration of virus alone was prepared and used as an indicator of virus infectivity in the absence of ink. Likewise, dilutions of the ink were inoculated on PK-15 cells to test for toxicity.

Results

On postinoculation day (PID)-2 in the first trial, the donor pig inoculated with the Ames virus had inappetence, and by PID-3 it had a temperature of 105.4° F. On PID-7 the pig's blood contained 19,500 viral PFU per ml. At this time the infected pig was slapped with the tattoo instrument.

The three recipient pigs remained healthy after being slapped with the tattoo instrument. Hog cholera virus was not isolated from the blood of these pigs 7 and 27 days later. Hog cholera antibodies were not detected in the serums of the pigs 27 days after being slapped with the instrument. When the immunity of the pigs was challenged with the Ames virus, all contracted HC, and virus was isolated from their blood 9 days later.

In the second trial the donor pig was slapped on PID-6 with the tattoo instrument. At this time its blood contained 7,400 viral PFU per ml.

The first recipient pig became ill 1 day after being slapped with the tattoo instrument. The remaining two pigs became ill 3 and 4 days, respectively, after being slapped with the instrument. Hog cholera virus was isolated from spleens taken from the pigs at necropsy 10 days postexposure. In each case the inoculated PK-15 cell cultures had viral PFU too numerous to count.

Virucidal activity was not demonstrated with the tattoo ink. In fact the virus titer was slightly higher when titrated in the presence of the ink (table 1). High concentrations (1:10) of the ink did not produce visible toxic effects on the cell cultures.

Table 1. - Titration of hog cholera virus with and without tattoo ink

	Tenfold Dilutions				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
With Ink	4/4[1]	4/4	4/4	4/4	3/4
Without Ink	4/4	4/4	4/4	4/4	0/4

[1] Numerator - Cell cultures from which hog cholera virus was isolated; denominator - Cell cultures inoculated with virus dilutions.

Discussion

Transmission of HC virus with the tattoo instrument was accomplished with the donor pig having the lowest blood-virus titer (second trial) but not with the donor pig having the highest virus titer (first trial). Transmission of HC virus in the second trial may have been related to the procedure that was followed; for example, the donor pig was slapped prior to slapping each recipient pig increasing the chance of transmission.

When PK-15 cell cultures were inoculated with tenfold dilutions of the tattoo ink, ink particles settled and attached to the cells. While visible macroscopically at the 1:10 and 1:100 dilutions, the particles were observed only microscopically at higher dilutions. Vigorous agitation did not dislodge the ink particles from the cells. It is suggested that the higher virus titer occurring in the presence of ink was related to an entrapment of virus by the ink particles, thereby affording protection to residual virus and preventing its dislodgement when washing the cell cultures.

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[3] Everhot Manufacturing Company, Maywood, Ill.

In addition to transmission of HC virus, the slap tattoo instrument has been shown to transmit *Erysipelothrix rhusiopathiae*.[4] Considering the disease transmission potential of the instrument, it should not be used on swine returning to the farm. A slap tattoo instrument has been used in Canada for the identification of slaughter swine.[5] Perhaps the instrument has some application for this purpose.

Acknowledgments

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[4] Harrington, R. 1972. Personal communication

[5] Miller, R. R. 1972. Animal identification in Canada. Proc. Annual Meeting Livestock Conservation, Inc.: 14-15.

PERSISTENCE OF PASSIVELY ACQUIRED HOG CHOLERA ANTIBODIES. Stewart, W. C., Snyder, M. L., Kresse, J. I., and Carbrey, E. A. (Project Report)

Summary

Eight pigs that were serologically negative for hog cholera (HC) and weighed 36 to 52 pounds were given 40 ml of anti-HC serum. Serum specimens were obtained from the pigs at intervals of 7 days and examined for the passively acquired antibodies by the fluorescent antibody serum neutralization test (FASNT). Low antibody titers were detected in one half of the pigs 140 days later.

The passively acquired antibodies declined at a fairly constant rate. The half life of the antibodies was found to be 21.3 days.

Introduction

Hog cholera antibodies may be detected by the FASNT, however, this procedure does not distinguish between actively and passively acquired antibodies. The method by which the antibodies are derived is quite important when an attempt is made to determine the significance of serologic titers.

Previously, it was shown by Coggins[1] that colostral antibody titers persisted in pigs for as long as 3 to 3.5 months provided the pigs' dam had a HC antibody titer of $\log_{10} 3.0$ or greater at the time of farrowing. Serologic studies were performed at this laboratory on pigs whose dams originated from a HC infected premise and it was shown that the colostral titers required a slightly longer period to disappear (unpublished).

Anti-HC serum has been used extensively in the treatment of HC and as supportive therapy for other diseases. Information as to how long these passively acquired antibody titers persist has not been available. The purpose of this investigation was to determine the length of time necessary for the antibodies acquired by the administration of anti-HC serum to disappear.

Materials and Methods

Pigs—Each of eight pigs ranging in weight from 36 to 52 pounds was inoculated subcutaneously with 40 ml of anti-HC serum.[2] Pigs 1 through 4 were inoculated behind the ear and pigs 5 through 8 were inoculated in the flank. The pigs were shown to be serologically negative for HC antibodies at the 1:4 serum dilution before they were inoculated with the anti-HC serum.

Serums—Clotted blood specimens were collected from the pigs at intervals of 7 days for 20 weeks, and the serums were examined by the FASNT.

Serum Neutralization Tests—Four-fold dilutions of the serums were prepared and mixed with equal volumes of HC virus suspension containing 2,500 plaque forming units (PFU) per 0.1 ml. After incubating the serum-virus mixtures at 37 C. for 1 hour, 0.2 ml quantities were inoculated on PK-15 cells. Following overnight incubation the serum neutralizing titers were determined as previously described.[2]

Results

The anti-HC serum administered to the pigs was found to have an antibody titer of $\log_{10} 3.6$. Seven days postinoculation (DPI) HC antibody titers of 2.4 were detected in seven pigs and one pig had a titer of 1.8. The titers declined at a linear rate, and the first pig became serologically negative 126 DPI. When the investigation was terminated 140 DPI, four pigs had titers of 0.6, three pigs were serologically negative, and one pig had died after a blood specimen was taken 119 DPI. The serologic titers of the pigs are shown in table 1.

The half life of the passive antibodies was calculated by regressing the log titers against DPI and was estimated to be 21.3 days with a 95 percent confidence interval of 20.0 to 22.9 days. Mean log titers were also calculated for

[1] Coggins, L. 1964. Study of hog cholera colostral antibody and its effect on active hog cholera immunization. Amer. J. Vet. Res. 25: 613-616.

[2] Affiliated Laboratories Corporation, East St. Louis, Ill.

Table 1.—Passive antibody titers occurring in swine after inoculation with 10 ml of anti-hog cholera serum

Pig (number)	Weight (pounds)	Days postinoculation																				
		0	7	14	21	28	35	42	49	56	63	70	77	84	91	98	105	112	119	126	133	140
1	50	0.0	2.4	1.8	1.8	1.8	1.8	1.8	1.8	1.2	1.2	1.2	1.2	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.0	0.0
2	36	0.0	2.4	1.8	1.8	1.8	1.8	1.8	1.8	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	0.6	0.6	0.6	0.6
3[1]	52	0.0	2.4	2.4	2.4	1.8	1.8	1.8	1.8	1.2	1.2	1.2	1.2	0.6	0.6	0.6	0.6	0.6	0.6	NS[2]	NS	NS
4	42	0.0	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.2	1.2	1.2	1.2	1.2	1.2	1.2	0.6	0.6	0.6	0.6	0.6
5	40	0.0	2.4	2.4	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.2	1.2	1.2	1.2	1.2	1.2	1.2	0.6	0.6	0.6	0.6
6	45	0.0	2.4	2.4	2.4	1.8	1.8	1.8	1.8	1.8	1.8	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	0.6	0.6	0.6
7	49	0.0	2.4	2.4	1.8	1.8	1.8	1.8	1.8	1.2	1.2	1.2	1.2	1.2	1.2	0.6	0.6	0.6	0.6	0.0	NS	NS
8	43	0.0	2.4	2.4	1.8	1.8	1.8	1.8	1.8	1.2	1.2	1.2	1.2	1.2	1.2	0.6	0.6	0.6	0.6	0.6	0.0	0.0

[1] Pig no. 3 died after the blood specimen was taken 119 days postinoculation.
[2] NS - No sample.

each DPI and plotted (fig. 1). These titers closely approximate the calculated regression line. A statistical difference in the rate of decline of the antibodies relative to the site of inoculation was not shown at the 0.05 level.
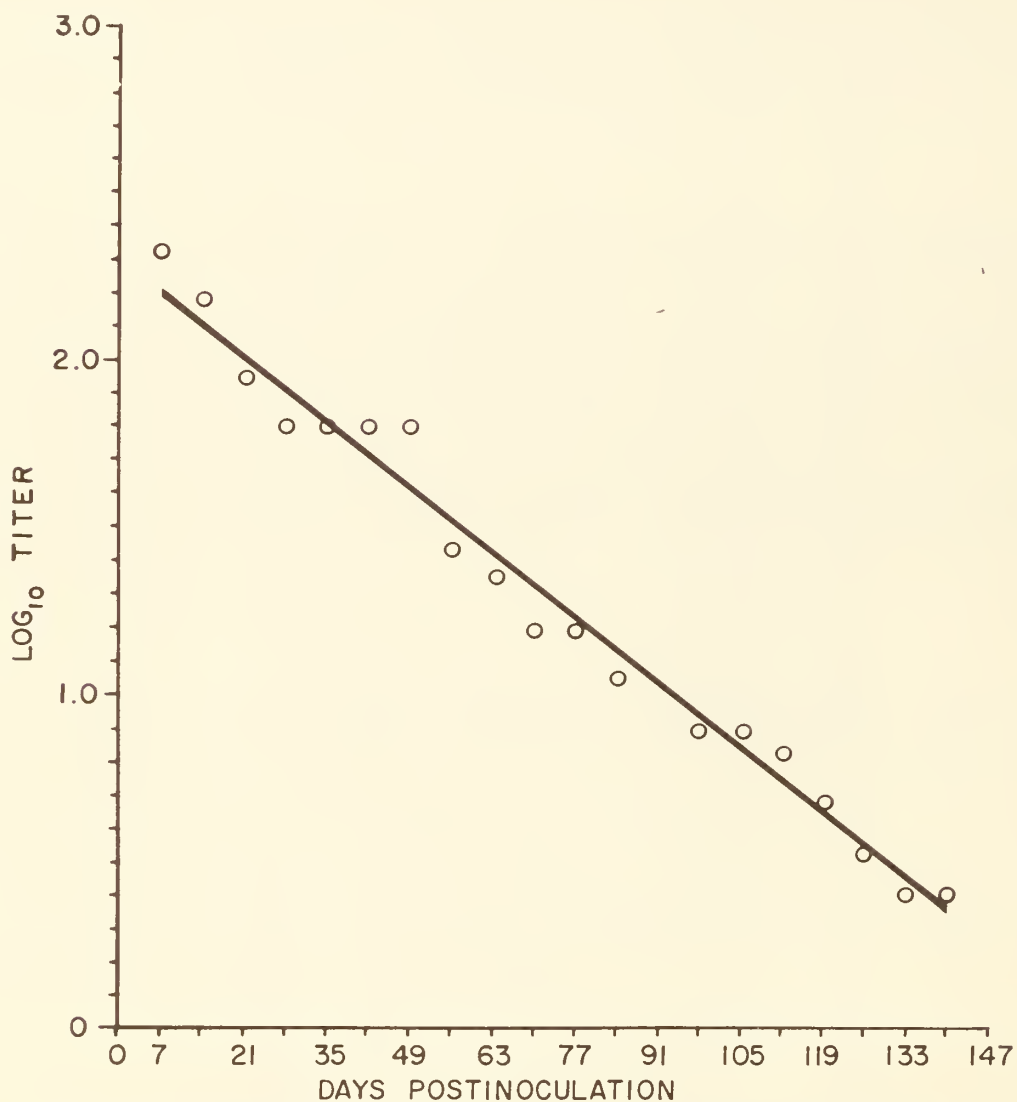
Figure 1.—Deviations of the mean antibody titers of 8 pigs from a constant rate of antibody decline.

Discussion

Moderate to moderately high antibody titers were detected in the swine 7 days after being inoculated with anti-HC serum, and the titers persisted in one-half of the pigs for 140 days. These results indicate that one must use caution when interpreting antibody titers in swine that have a history of being inoculated with anti-HC serum. In such cases it is recommended that a second serum sample be examined 21 to 28 days later to determine if the antibody titers have declined. A declination of the titers should occur with passively acquired antibodies. With

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actively acquired antibodies the titers should increase if infection occurred recently, otherwise the titers would remain constant.

The period of 140 days in which these passive antibodies were detectable is similar to the period in which colostral antibodies were found to persist and which was alluded to in the beginning of this report (unpublished).

The period of time that passively acquired antibodies will remain in a pig is dependent on several factors: (1) titer of the anti-HC serum, (2) quantity administered, and (3) size of the pig. In the present investigation the anti-HC serum had a high titer, and a large quantity was administered, approximately 1 ml per pound of body weight. It is believed that the results of this investigation represent the maximum response that will occur from the routine administration of anti-HC serum in the field.

Generally, the passive antibodies disappeared first in the larger pigs. For example, when the investigation ended, the pigs having antibody titers weighed less than 45 pounds. The weights of the pigs that had become serologically negative were 50, 49, and 54 pounds. Pig No. 3 had a borderline titer 119 DPI. Had the pig not died, it is believed that it would have reached a negative serologic status at 126 DPI.

Acknowledgments

[3]Carbrey, E. A., Stewart, W. C., Kresse, J. I., and Lee, L. R. 1969. Confirmation of hog cholera diagnosis by rapid serum neutralization technique. Jour. Amer. Vet. Med. Assoc. 115: 2201-2210.

Pregnant sows, vaccinated with attenuated or inactivated vaccines, were inoculated with a field strain of low virulent hog cholera (HC) virus to determine if transplacental infection occurred. Serologic responses were detected with the fluorescent antibody serum neutralization technique and transplacental infection was confirmed by isolating the virus from the offspring, using the fluorescent antibody cell culture technique (FACCT) and pig inoculation.

Transplacental infection did not occur in nine sows that had been vaccinated with attenuated virus and had an antibody titer of $\log_{10}$ 1.2 or greater when given injections of the field virus. One sow which was supposed to have been vaccinated, but lacked measurable antibody titer and died after infection, was shown to have transmitted the virus to its young *in utero*. In contrast, antibody titers were low or nondetectable in 12 sows that were vaccinated with the inactivated vaccine. When inoculated with the field virus, these sows had variable clinical responses, and in three of the six sows that produced litters, transplacental infection occurred.

Data obtained from the experiments provided information on the danger associated with inoculation of virus in vaccinated immune and vaccinated nonimmune pregnant females.

MYCOBACTERIOSIS

SODIUM BORATE FIXATION FOR HISTOPATHOLOGY. Miller, L. D. (Project Report)

Summary

The use of saturated sodium borate solution (SBS) for preservation of tissues for histopathologic study was investigated and it was found unsatisfactory. The purpose was to determine if SBS, now distributed for transport of specimens to the bacteriology laboratory, could replace neutral buffered 10 percent formalin as the preservative for histopathology specimens.

Introduction

Saturated sodium borate solution has long been recognized as a tissue macerating agent[1] and recently was found to be a suitable medium for transport of tuberculosis specimens to the bacteriology laboratory.[2] Its usefulness as a short-term preservative of tissues for histopathologic study was examined. Satisfactory results could have eliminated the need for specimen submission in 10 percent formalin.

Materials and Methods

Portions of guinea pig liver and spleen were allowed to stand in SBS or neutral buffered 10 percent formalin for 1 to 5 days. Tissue sections were prepared for histopathologic examination by routine laboratory procedures. In addition, three field submissions for tuberculosis examination were included for study. These specimens were removed from SBS and placed in 10 percent formalin for 24 hours before further processing. The suitability of SBS for routine use was evaluated by comparison of SBS preserved tissues to those fixed in 10 percent formalin.

Results

Diffusion of hemoglobin pigment into the borate solution was the first change noted and was present in all SBS specimens. The tissues held in SBS were less firm and more difficult to slice than those fixed in formalin.

Microscopically, the most prominent change in the SBS specimens was the disappearance of erythrocytes from the tissues. Other changes were pyknosis of cell nuclei, loss of cytoplasmic detail and alteration of staining characteristics (fig. 1) when compared to tissue fixed in 10 percent formalin (fig. 2). As expected the autolytic processes were progressively more severe as the holding time in SBS increased.

Conclusion

Saturated sodium borate solution was not suitable as a preservative for tissues intended for careful histopathologic study. Tissue autolysis was not presented. The necessary sterilization of specimens from field submissions precluded immediate processing. This delay is an additional disadvantage and would be particularly undesirable when carcass disposition at the packing plant is pending the results of histopathologic examination.

Acknowledgment

[1] Humason, G. L. 1962. Animal tissue techniques. W. H. Freeman and Co., San Francisco Calif. 10.

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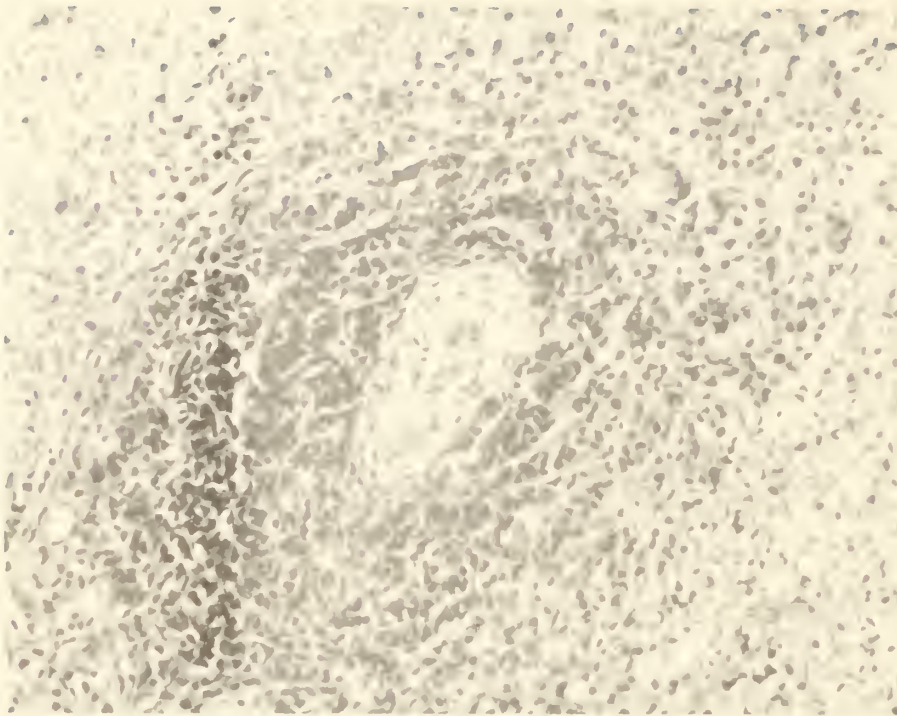
Figure 1.—Spleen held in SBS for 3 days. Compare to figure 2. Note pyknosis of cell nuclei, loss of cytoplasmic detail and the empty appearance of sinusoids in the red pulp. H & E 285X.
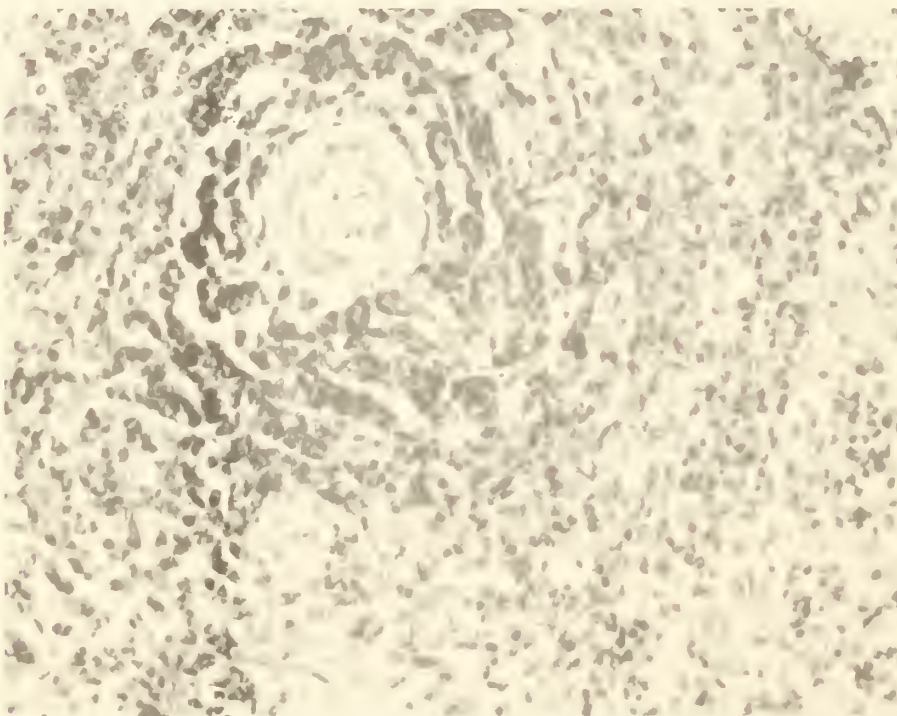
Figure 2.—Spleen held in neutral buffered 10% formalin for 3 days. Note the various cell types and structural detail not recognizable in figure 1. H & E 285X.

CASE REPORT-ISOLATION OF M. PARATUBERCULOSIS FROM A COW AT ROUTINE SLAUGHTER.
Richards, W. D. and Muhm, R. L. Proc. 72nd Ann. Mtg. USAHA, 1971: 518-522.
(Abstract of Published Report)

Mycobacterium paratuberculosis was isolated from granulomatous lesions from a 10-year-old cow consigned to a packer for routine slaughter. Histopathologic findings were compatible for Johne's disease even though caseation and calcification observed in lymph nodes was contrary to the classic histopathologic description of Johne's disease. Herrold's Egg Yolk Agar supplemented with mycobactin was the only one of eight laboratory mediums which supported the growth.

The diagnosis of Johne's disease is complicated by lack of specificity of skin test reagents and by the similarity of clinical signs to other disease conditions. The etiologic agent, Mycobacterium paratuberculosis, proliferates in the intestine and is frequently shed in the feces of the infected animal. Saprophytic acid-fast bacilli exist as a part of the normal flora of feces from healthy and infected animals and cannot be differentiated from pathogenic acid-fast bacilli on the basis of cellular morphology. For this reason, the microscopic demonstration of acid-fast bacilli in stained smears of feces is not generally considered confirmative for the diagnosis of Johne's disease.

Recent developments in culture methods have made possible the successful isolation and identification of M. paratuberculosis from feces or tissue specimens. M. paratuberculosis is dependent upon mycobactin, an extract of M. phlei for multiplication in vitro. This is a report of an isolation at the Veterinary Services Diagnostic Laboratory of M. paratuberculosis from tissues collected from a cow in which Johne's disease was not suspected. The use of this medium with each routine mycobacteriology case could provide an effective surveillance method for Johne's disease in cattle with granulomatous leisons regardless of the reason for slaughter.

MECHANISMS OF CHANGES IN SERUM LIPID-LIPOPROTEIN PROFILES IN INFECTIOUS DISEASE MYCOBACTERIUM BOVIS INFECTION IN RABBITS. Thoen, C. O., Karlson, A. G., and Ellefson, R. D. Mayo Clinic Proceedings. Vol. 47. 1972, pp. 270-272. (Abstract of Published Report)

Elevations of serum cholesterol levels occurred in *Mycobacterium bovis* infected rabbits. In this study, a radioactive precursor of cholesterol was followed to obtain information on the synthesis and disposal of serum cholesterol in rabbits before and after infection.

The incorporation of $2\text{-}^{14}C$-mevalonic acid into serum cholesterol and the disposal of $2\text{-}^{14}C$-cholesterol from serum were determined for each of seven rabbits before, and 23 days after, infection with *Mycobacterium bovis*. In comparison to the preinfection measurements of radioactive cholesterol, postinfection measurements indicated that the disposal of serum cholesterol was impaired in infected rabbits.

The disposal of cholesterol from circulating lipoproteins is largely a function of liver via biliary excretion and bile acid synthesis and excretion; however, an impairment of cholesterol disposal is suggestive of an impairment of liver function. Although visible lesions in liver became extensive after infection with *M. bovis*, the extent of these would not seem to have been sufficient to suppress cholesterol disposal to the extent that was observed. Impairment of the hepatic disposal of cholesterol, then, might have resulted in part from undetected, widespread damage to certain organelles of the hepatocytes, or perhaps from a widespread inhibitory effect of a toxic substance.

Regardless of the form of the underlying mechanism, it is clear that the hypercholesteremia that developed as a result of *M. bovis* infection arose from an impairment of cholesterol disposal and not as a result of an accelerated synthesis and secretion of lipoproteins into blood.

Although some progress has been made in the understanding of alterations of the serum lipid-lipoprotein profiles in certain metabolic conditions, little information is available on serum lipid-lipoprotein profiles in infectious disease. The objectives of this study in rabbits were (1) to study systematically the effects of certain bacterial infections on the serum lipid-lipoprotein profile, (2) to determine whether changes in the profile persist after disease, and (3) to explore the diagnostic and prognostic value of lipid-lipoprotein profile in infectious disease.

Serum phospholipid, cholesterol, and triglyceride levels became elevated in rabbits during the courses of four bacterial infections. The increases of serum lipids were greater in the acutely ill rabbits that subsequently died than in the animals that survived.

Lipoprotein analysis of whole serum and of ultracentrifuge fractions of serum by paper electrophoresis revealed increases of very low density components associated with concomitant decreases of low density and high density components. The changes of the serum lipid-lipoprotein profile that developed during disease were reversible; during recovery, normal lipid-lipoprotein profiles were reestablished.

These experiments show that bacterial infections can influence lipid and lipoprotein metabolism in rabbits. The liver plays a central role in the regulation of the blood lipid-lipoprotein profile; however, the changes that occurred in the blood lipid levels of infected rabbits usually were not reflective of changes in liver lipid levels. The data suggested, however, that alterations occurred in the synthesis or in secretion of lipids from the liver in certain diseases.

A toxic substance affecting all liver cells might have been responsible in part for the massive biochemical changes that occurred, but the nature of the changes suggested that they arose mainly from impairment of the mechanisms by which lipids normally would be cleared from circulating lipoproteins.

THE SIGNIFICANCE OF MYCOBACTERIUM TUBERCULOSIS INFECTION IN CATTLE: A LITERATURE REVIEW. Harrington, R., Jr. and Thoen, C. O. (Literature Review)

Summary

Mycobacterium tuberculosis is responsible for most of the tuberculosis in man and has been shown to sensitize cattle to mammalian tuberculin. However, cattle infected with this bacillus appear to represent little danger to domestic animals and man. Since it is not possible to distinguish sensitization caused by *M. tuberculosis* from that caused by *M. bovis*, the importance of human-type infection in cattle must be considered in the campaign to eradicate bovine tuberculosis from the United States.

Introduction

Originally it was believed that the tubercle bacillus responsible for disease in man and animals was the same microorganism (9). Smith (90), in 1896 and 1898, established that human and bovine tubercle bacilli differed in growth characteristics on culture media and in pathogenicity for the rabbit and cow.

As early as 1899, it was reported that a tuberculin negative cow converted to tuberculin positive after being attended by a tuberculous herdsman (87). The tubercle bacillus responsible for the infection in the herdsman and in the cow was not identified; therefore, one cannot determine from this report whether the human type tubercle bacillus was responsible for the tuberculin conversion in the cow.

In 1901, it was demonstrated that cattle were relatively resistant to infection with human tubercle bacilli, even when the microorganisms were injected intravenously (40). This led some to believe that tuberculosis in man caused by the human type tubercle bacillus was not transmissible to cattle, and that tuberculosis in cattle could be prevented by vaccination with the human strain. Later this theory was disproved when it was reported that the human type tubercle bacillus was capable of infecting cattle (72).

The terms "human" and "bovine" tubercle bacilli (respectively, *Mycobacterium tuberculosis* and *Mycobacterium bovis*) only indicate the primary host for each type; they are not indicative of host specificity (39). Each type is capable of producing progressive disease in a large number of hosts, including man and some farm animals, household pets, and wild animals.

Differentiation Between M. tuberculosis and M. bovis

M. tuberculosis is slightly less virulent than *M. bovis* for most species and practically nonpathogenic for some (18). Man, monkeys and guinea pigs are highly susceptible to *M. tuberculosis* whereas cattle, sheep, goats, rabbits and birds (with the exception of the parrot family and possible canaries) are very resistant (74). In the laboratory *M. tuberculosis* is differentiated from *M. bovis* by the failure of the former to produce progressive fatal disease in rabbits in contrast to the progressive disease caused by *M. bovis* (17). Lesions in cattle resulting from infection with human type bacilli, when present tend to be localized (15,28,29,49,50,105).

M. bovis is more virulent than *M. tuberculosis* even in experimental animals such as the guinea pig, which is susceptible to both types (18). In man and monkey bovine tubercle bacilli are no less virulent than human type tubercle bacilli once the infection is established.

Human and bovine tubercle bacilli are aerobes and will not grow in the absence of oxygen. Both strains grow best at 37°C, but do not grow below 30°C. or above 42°C. (8). Four to six weeks are generally required for abundant growth, although colonies may appear in less than four weeks.

For primary isolation, both types require enriched medium which contains eggs or serum proteins. Dyes may be incorporated in the medium to inhibit the growth of contaminants (38). After primary isolation, strains of *M. tuberculosis* grow well on synthetic medium containing glycerol, asparagine, magnesium citrate and certain inorganic salts. Usually the human strains grow more abundantly than the bovine strains on culture medium. Human type tubercle bacilli tend to grow better when glycerol is added to the medium whereas bovine type bacilli appear to be inhibited (79).

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[1] Numbers in parenthesis refer to References at end of this paper.

Infections in man and animals caused by human and bovine type tubercle bacilli cannot be differentiated by means other than isolation and identification of the microorganism (23). It is of epidemiologic importance to identify infections in man caused by bovine type tubercle bacilli when it occurs; similarly, it is important to identify infections in cattle caused by human type tubercle bacilli as a possible cause of disease and tuberculin sensitivity.

Smith (90), as mentioned previously, was the first to show that there were morphological and cultural differences between human and bovine tubercle bacilli. *In vitro* tests that can be used to differentiate human and bovine tubercle bacilli are: (1) niacin production (23,38), (2) nitrate reduction (23, 103), (3) thiophen-2-carboxylic acid hydrazide sensitivity (5,36,76,104), (4) nicotinamide sensitivity (23,31), and (5) nicotinamidase activity (6,41,54). The differential features of *M. tuberculosis* and *M. bovis* are summarized in table 1.

Table 1.—Differential characteristics of *M. tuberculosis* and *M. bovis*

	M. tuberculosis	M. bovis
Pathogenicity:		
Guinea pig	+++	+++
Rabbit	+	++++
Niacin Production	+	—
Nitrate Reduction	+	—
Nicotinamidase	+	
Inhibited by:[1]		
TCH[2]	—	+
Nicotinamide	+	—
Glycerol[3]	—	+

[1] Inhibited = (+), resistant = (−).
[2] TCH = Thiophen-2-Carboxylic Acid Hydrazide.
[3] On original isolation, the presence of glycerol in the medium enhances the growth of *M. tuberculosis* but inhibits the growth of *M. bovis* in most cases.

Differentiation by the Tuberculin Test

Much has been said about the specificity of the tuberculin test during the progress of bovine tuberculosis eradication programs throughout the world. The tuberculin test has been one of the most important means of diagnosing tuberculosis in cattle.

Koch was the first to demonstrate the activity of tuberculin in 1880 (18) and during this same year Guttmann performed the test on cattle. Large scale trials soon demonstrated the usefulness and limitation of the tuberculin test.

If the bovine tubercle bacillus was the only microorganism capable of sensitizing cattle to tuberculin, tuberculin testing would be relatively simple because one could use a tuberculin of sufficient potency to insure the detection of all but a small number of tuberculous animals. Present evidence indicates that tuberculin sensitivity in cattle may be caused by infection with *M. tuberculosis* (15,40), *M. paratuberculosis* (11,35,98), *M. avium* (15,43,57,98), non-pathogenic mycobacteria (24,63) and possibly other acid-fast organisms (1,48). However, the primary cause for non-specific responses to mammalian tuberculin in cattle is thought to be infection with the avian or human tubercle bacillus (15,18,39).

To decrease the number of non-specific responses to tuberculin in cattle, it has been recommended that dilute tuberculin be used (4,51,52,64,85,110). Field trials proved this to be unsatisfactory because a number of animals with tuberculous lesions did not respond to the test (10).

Lesslie (46) and Pavlas (65,66) studied the specificity of Purified Protein Derivative (PPD) tuberculins prepared from avian, bovine and human strains on calves sensitized with heat-killed mycobacteria. The reactions with bovine and human PPD were not significantly different. These investigators also reported that PPD tuberculin, even when diluted, could not be used to differentiate sensitivity induced in cattle by human and by bovine type tubercle bacilli. Investigation on calves experimentally infected with *M. bovis* and with *M. tuberculosis* demonstrated that all animals so infected had a positive response to mammalian tuberculin after 2-4 weeks (86). Finally, Kuslys (42) reported that in a herd free of bovine type infection, cattle may respond greater to mammalian tuberculin if spontaneously infected with human type tubercle bacilli.

From these reports it is evident that one cannot differentiate infections in cattle caused by human type tubercle bacilli from those resulting from bovine type tubercle bacilli using diluted or undiluted tuberculins.

Susceptibility of Cattle to M. tuberculosis

In 1899, a case was reported in which a tuberculin negative cow, after being attended in a separate stable by a tuberculous patient, became tuberculin positive (32). At slaughter, extensive tuberculous lesions were present in the parenchyma of the lung and pleura of this animal. No bacteriological examination was made of the lesions from the patient or animal; therefore, it is impossible to say what organism produced these lesions.

In 1901, it was found that calves experimentally infected with a *M. tuberculosis* isolate from human patients with disease produced no clinical signs (40). Although no tuberculous lesions could be demonstrated on post mortem examination, *M. tuberculosis* was isolated from a small suppurative focus at the site of inoculation in 1 of the 19 animals inoculated.

In 1913, naturally acquired tuberculosis was reported in a calf which had been raised on milk from cows vaccinated by intravenous inoculation with human type tubercle bacilli (21). The post mortem examination of this animal showed slight tuberculous lesions in the mesenteric lymph nodes. Human type tubercle bacilli were isolated from these lesions. Studies have shown that cattle naturally infected with human type tubercle bacilli may excrete virulent bacilli in the milk from apparently normal udders (34,53,56,82).

Hibma (27) in 1933 found a 4-year-old cow that gave a positive response to the tuberculin test. This animal was on a farm free of bovine tuberculosis. No cattle had been added to the herd and no swine or poultry were present on the premises. At slaughter, tuberculous lesions were observed in the bronchial and mediastinal lymph nodes. The organism isolated from the lesions had some characteristics similar to *M. tuberculosis*. No attempt was made to type the isolate by animal inoculation test.

Henderson (25) reported tuberculous lesions in bronchial lymph nodes in a cow from a herd in which tuberculin responses occurred. Since no type differentiation was mentioned, it is now known if this, in fact, was an infection caused by human type tubercle bacilli.

Reid (71) reportedly isolated human type tubercle bacilli from a suspension of parotid, pharyngeal and submaxillary lymph nodes from a heifer in which no tuberculous lesions could be demonstrated at slaughter.

Crawford (12), in an attempt to determine the susceptibility of cattle to the human type tubercle bacillus, injected 23 animals subcutaneously with this strain. None of the animals so injected had lesions on post mortem examination.

Hillermark (28), on five occasions, isolated human type tubercle bacilli from cattle reacting to tuberculin. No lesions of tuberculosis could be demonstrated macroscopically either in the lungs, regional lymph nodes or in the mesenteric nodes. He later isolated human type tubercle bacilli from 12 which had reacted to bovine tuberculin (29). Seven of the animals had no macroscopic tuberculous lesions at slaughter. In five, localized caseous and calcified tubercles were observed.

Kuslys (42) in 1950 reported that human type tubercle bacilli were isolated from a bull 1 year of age from a herd in which bovine tuberculosis was apparently eradicated.

In 1953, Fromm and Wiesmann (19) isolated human type tubercle bacilli from eight cows. Lesions were detected macroscopically at slaughter in seven of these animals.

Henderson and Stenius (26) evaluated the tuberculin test in cattle infected with human type tubercle bacilli. They found tuberculin sensitivity occurred about 2 months after infection. Responses were small and not stable. Investigations by Plum (69) indicated that cattle infected with human type tubercle bacilli may become tuberculin positive, although no lesions may be demonstrated at slaughter. He also found that tuberculin responses usually disappear when the human source is removed.

Griffith (22) reported a case in which tuberculous lesions were present in a calf which had previously reacted to the tuberculin test; human type tubercle bacilli were isolated from the lesions. A similar case was reported by Brook (7) in which the human type tubercle bacillus was isolated from the retropharyngeal lymph glands of a cow.

Neilson and Plum (64) made a study of cattle exposed to humans with open pulmonary lesions due to the human type tubercle bacilli and found that cattle may become readily sensitized to tuberculin, but lesions are rarely demonstrated on post mortem examination.

Nemoto (59) and associates made cultures of tissues from a cow which was a tuberculin reactor without visible lesions. They also examined the owner and isolated tubercle bacilli having the character of *M. tuberculosis* from both. Previous to this, lung tissue and bronchial lymph nodes from cattle reacting to the tuberculin test were cultured by Plum who isolated human type tubercle bacilli from 4 of 644 specimens (68).

Hirato and Shimizu (30) bacteriologically examined 33 tuberculin reactors which had no tuberculous lesions at slaughter. Human type tubercle bacilli were isolated from lymph nodes in six of these animals.

An interesting case was reported by Sugimura (96) and associates in which human type tubercle bacilli were isolated from a superficial hemorrhagic area in the lung of a cow which had previously reacted to tuberculin. No typical tuberculous lesions were observed in the animal.

An 8-year study was conducted by Lesslie on 1,990 specimens from tuberculin reactor cattle (44). Human type tubercle bacilli were isolated from 12 of the reactors; however, on post mortem examination only 4 of the animals had small lesions in the respiratory or alimentary system.

Another interesting study was conducted by Illes (33) on the relationship between tuberculosis in man and cattle on small farms. From 1950-1959, approximately 10,000 cattle were tuberculin tested with 11.8 percent having a positive response. Contact of reactors with tuberculous persons was reported on 264 of 401 farms. He concluded that the high percentage of reactors was due to contact between the cattle and persons infected with human type tubercle bacilli.

Shibata (86) artificially infected 12 calves with human and with bovine tubercle bacilli and found that all calves infected with bovine type tubercle bacilli had typical lesions in the lymph nodes and organs whereas those infected with human type tubercle bacilli had no lesions. Human type tubercle bacilli were isolated from five of the eight animals infected with this strain.

The only evidence which suggests that human type tubercle bacilli may produce lesions similar to the bovine tubercle bacillus is the investigation by Spryszak and Zorawski (91). They reported that lesions produced by the two types were similar; however, it must be remembered that the animals were experimentally infected. Cattle naturally infected with human type tubercle bacilli showed no lesions macroscopically (77); and when lesions did occur, they were small and localized (99).

In order to elucidate the source of infection with human type tubercle bacilli in cattle, human contacts should be examined and bacteriologic studies made (37). As early as 1932 it was believed that man served as the source for cattle infected with human type tubercle bacilli (26). Moreover, the work of Plum (70) and Stenius (92) showed that when a person with pulmonary tuberculosis (caused by the human tubercle bacillus) was allowed to take care of tuberculin negative cattle, the animals become sensitized to tuberculin. More recently a number of authors have reported that man was the source for human type tubercle bacillus infection in cattle (20,42,44,60,73,75,82,94,107).

From the literature cited, it appears that cattle usually have a striking resistance to human tubercle bacilli and that incidence of human type infection is apparently greatest in stabled cattle (78). Although infection with *M. tuberculosis* may occur, the organism rarely appears to be responsible for progressive disease in cattle.

Prevalence

Tuberculosis in cattle caused by human type tubercle bacilli has been observed for many years and has been reported occasionally. Compared to infection by *M. bovis* in the bovine, it is considered of little importance (87). However, since *M. bovis* infection in cattle reached a low level, more significance must be given to the problem of infection with human type tubercle bacilli (37,93).

In Japan, Watanabe (106) made a bacteriological examination of 88 cases of tuberculosis during the years 1939-1941 and found that 3.4 percent were infected with human type tubercle bacilli. Plum (68) in Denmark (1952) examined tissues from tuberculous cattle and found that 0.35 percent were infected with human type tubercle bacilli. Lesslie (45) in England and Wales (1959) made a similar study of 3,934 specimens, 0.18 percent of

which contained human type tubercle bacilli. Meyn (51) in Austria (1961) reported on 167,000 cattle reacting to mammalian tuberculin. Of this number, 1.26 percent were infected with human type tubercle bacilli. Schneider (83) in Switzerland (1963) examined 65 specimens from tuberculin reactors and found that 0.34 percent were infected with human type tubercle bacilli. Pavlas (67) (Czechoslovakia, 1964) examined 10,239 tuberculin reactors; 0.33 percent were infected with human type tubercle bacilli. During this same year, another study was done by Simek (89) in Czechoslovakia, who examined tissues from 195 tuberculous cattle. The number infected with human type tubercle bacilli was 1.5 percent. In Russia, Odarenk (62) typed mycobacteria isolated from cattle and found that 2 percent were human type tubercle bacilli. Other workers also have occasionally isolated human type tubercle bacilli from cattle (3,16,88,95). At the present, data are not available on the occurrence of human type infection in cattle in the United States; a general impression is that it occurs infrequently (109).

The actual prevalence of cattle infected with human type tubercle bacilli cannot be determined from the literature because only a small segment of the cattle population in the various countries has been studied and the data are insufficient. Therefore, only a general estimate of the frequency of infection can be made.

Significance

It has been reported that cows infected with human tubercle bacilli may excrete virulent bacilli in the milk which may be dangerous to man and other cattle (9,21,58,81,82). However, it appears unlikely that milk is commonly responsible for animal-to-animal or animal-to-man transmission of *M. tuberculosis* (15,55,63).

The problem of greatest significance is the sensitization of cattle to mammalian tuberculin as a consequence of exposure to human type tubercle bacilli from human sources. In Finland, Stenius (93) after a comprehensive investigation showed that the high percentage of reactors, which was higher than the percent lesions found at slaughter, was largely due to infection with *M. tuberculosis*. Tajima (97) emphasized that infection of cattle with human type tubercle bacilli played an important role in the occurrence of "no visible lesion reactors" in Japan. Fromm and Wiesmann (18) found in nine herds previously negative to the tuberculin test that the occurrence of reactors was caused by exposure or by infection with human tubercle bacilli. McKinstry and Blampied (50) reported on six herds in the Jersey Islands over a 5-year period in which tuberculin responses were traced to tuberculous individuals. They found that 50 to 100 cattle exposed to tuberculous persons reacted to the tuberculin test. In this region which raised Jersey cattle for export, many of the laborers came from Brittany in France where tuberculosis was a serious problem in the human population. A regulation was passed that all workers from Brittany who were employed in caring for cattle must be free of tuberculosis. Ullmann (100) describes a herd in which 39 of 75 cattle reacted to the tuberculin test. It was shown that the herdsman and milker had tuberculosis. A similar report was made by Scholz (84). Finally, Endo (14) and associates reviewed post-war studies of bovine tuberculosis in Japan and found that the majority of the tuberculin reactors had no visible lesions at slaughter and they concluded that infection with human type tubercle bacilli was responsible for the tuberculin responses.

It has been said that infection of cattle with human type tubercle bacilli is of little importance and that animals so infected may remain in the herd without danger (19). One reason for this point of view is that progressive disease cannot be produced in the bovine by human tubercle bacilli. Bacteriologic investigations indicate that human type tubercle bacilli may remain alive for extended periods in the tissues of cattle naturally infected even if there are no gross lesions (80). Therefore, such infection should not be ignored.

Generally, human type infection in the bovine leads to sensitization to mammalian tuberculin which is of short duration if the human source is removed (47,61,92,94). Lesions rarely occur (51,108), and when present tend to be localized (13,102). When tuberculin sensitization in cattle occurs, and definite exposure to bovine type tubercle bacilli is unlikely or cannot be proven, consideration should be given to infection with human type tubercle bacilli.

In many instances, public health laws concerning tuberculosis were passed primarily to guard against direct contamination of milk with human tubercle bacilli (2). In the United States, approximately 50,000 new cases of tuberculosis occur each year in man (101). With the decline in *M. bovis* infection in cattle and the occurrence of large numbers of human cases, it should be emphasized that the importance of *M. tuberculosis* in sensitizing animals may be a factor in accomplishing the eradication of tuberculosis in cattle.

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PASTEURELLOSIS

THE PRODUCTION AND STANDARDIZATION OF TYPING ANTISERA FOR <u>PASTEURELLA MULTOCIDA</u>.
Angus, R. D., Kelley, D. J. and Pietz, D. E. (Project Report)

Summary

Antisera for identifying *Pasteurella multocida* serotypes 1, 3, 4 and 5 were prepared and evaluated using established procedures. Standardized antisera for the identification of these four serotypes of *P. multocida* are now available from the Biologic Reagents Section, Veterinary Services Diagnostic Laboratory, Ames, Iowa, and may be requested through the APHIS Veterinarian in Charge of each State.

Introduction

Bacterial infection of poultry by *P. multocida* produces a disease syndrome known as fowl cholera. This disease is relatively common in poultry and vaccines of various types are commonly used to reduce the economic loss and disease incidence.

In studying fowl cholera and related problems associated with vaccine use, it was found that *P. multocida* has several immunogenic types.[1] The ability to properly determine the serotype of a given *P. multocida* culture is often of considerable importance.[2] A source of standard typing antisera was considered an important second step after the initiation of a training program for laboratory personnel doing poultry diagnostic work.[3] Such training is now being given by the Veterinary Services Diagnostic Laboratory, Ames, Iowa.

This project was in response to the need for gaining competence in the production and standardization of *P. multocida* typing antisera by the Biologic Reagents Section of Veterinary Services Diagnostic Laboratories prior to routine antisera production activities.

Materials and Methods

Cultures.—The following cultures of *P. multocida* were obtained from the Diagnostic Bacteriology Section and used in producing and evaluating antisera: X-73 (serotype 1), P-1059 (serotype 3), P-1662 (serotype 4), and P-1702 (serotype 5).

Antisera.—Typing antisera were prepared by procedures previously reported[1][3] except for the following variations: (1) Each cell suspension was homogenized with oil for eight 30-second periods, with a 2-minute time interval between each homogenization period, and (2) both male and female chickens were used.

Precipitation test.—The agar gel double diffusion test was conducted as previously reported.[1]

Results

A total of twelve adult chickens were injected with antigen prepared from each of the four serotypes. The total and average yield of antisera are given in table 1. A pool of antisera was made from the 12 chickens injected with each antigen and submitted to the Diagnostic Bacteriology Section. The quality of each pool was evaluated by

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[2] Heddleston, K. L. Prevention and control of fowl cholera. Proc. 20th Western Poultry Disease Conference and 5th Poultry Health Symposium, pp. 75 (March, 1971). Agricultural Extension Service, University of California, Davis, Calif. 95616.

[3] Blackburn, B. O. Pasteurella serotyping (Project Report). Developmental studies and laboratory investigations conducted by diagnostic services, Veterinary Services, Ames, Iowa, Fiscal Year, 1971. APHIS 91-2 (May 1972) Animal and Plant Health Inspection Service, U.S.D.A., Ames, Iowa 50010.

testing against the homologous and heterologous antigens as previously recommended.[3] The evaluation results are recorded in table 1.

Although the degree of reaction of each composite serum with its homologous antigen was very weak to weak, there was a considerable variation between the degree of reaction of the serum of each chicken. Therefore, the poor reacting sera were discarded and the balance pooled and evaluated. The results are recorded in table 2.

Table 1. – The quantity and quality of each antiserum produced

| Serotype | Antisera yield (mls) | | Evaluation results | |
	Total	Average/chicken	Homologous[1]	Heterologous[2]
1	368	30.6	weak	none
3	367	30.6	--------do--------	Do[1]
4	267	22.2	very weak	with type 3 antigens
5	398	33.1	poor to very weak	none
TOTAL	1400	29.1		

[1] Degree of reaction.
[2] Cross reactions.

Table 2. – The quantity and quality of each useable antiserum

| Serotype | Number of chickens | Final useable antiserum yield (mls) | | Evaluation results | |
		Total	Average/chickens	Homologous[1]	Heterologous[2]
1	6	163	27.1	Good to excellent	none
3	11	292	26.5	--------do-------	none
4	5	99	19.8	Good	Minimal (2) cross reactions with type 3 cultures
5	6	147	26.2	Fair	none
Total	28	711	25.4		

[1] Degree of reaction.
[2] Cross reactions.

Discussion

These results were in agreement with previous reports relative to (1) the great variation of response between chickens inoculated with the same antigen; (2) the problem of cross reactions of strain P-1662 (serotype 4) with various cultures of serotype 3; and (3) the low antigenic response to strain P-1702 (serotype 5). [1,3]

The total and average yield of useable antiserum was somewhat lower than one would expect for routine production as a portion of each serum was used to prepare a pool for the preliminary evaluation tests.

The sera from about half of the chickens inoculated with antigens of serotypes 1, 4 and 5 were not satisfactory; therefore, if large volumes of these antisera are required, it would be advisable to conduct investigations for improving production methods.

Competence in producing antisera for typing *P. multocida* has been achieved by the Bacterial Reagents Unit of Biologic Reagents Section, Animal Health Diagnostic Laboratories, NADL.

Reference and typing antisera are now available, and may be requested through the APHIS Veterinarian in Charge of each State.

Acknowledgments

PESTICIDES

A BIOLOGICAL PRE-SCREENING SYSTEM FOR INSECTICIDES AND PESTICIDES.
Mote. R. F., Gigstad, D. C., and Muhm, R. L. (Project Report)

Summary

A biotic pre-screening test for pesticides using *Drosophila melanogaster* (a fruit fly) was described. Fly mortality indicated the need for additional chemical analysis. Colonizing methods and testing procedures were included. Median lethal (LD_{50}) dosages of several pesticides for a selected strain of fruit fly were calculated.

Introduction

There has been a steadily increasing demand for analysis of feed samples suspected of containing pesticides or other lethal materials. The use of a bioassay to study such samples has been investigated.[1][2] *Drosophila melanogaster* have been used to detect insecticide residues.[3][4] In this study, the susceptibility of a selected strain of long-lived flies, non-resistant to calculated dosages of various pesticides was evaluated.

Materials and Methods

Selecting the species.—Evaluations were made on six strains of *Drosophila* that had been laboratory cultured from 7 to 30 years and demonstrated little or no resistance to insecticides or pesticides. From these six, one strain was selected which had prolonged starvation characteristics and little genetic variation other than eye color.

Colonizing the flies.—Flies were colonized on agar media consisting of 1/4-cup agar to 1,000 ml of water brought just to the boiling point. To this 1/4-cup, brewer's yeast, 1/2-cup cornmeal and 2/3-cup molasses were added.[5] Prolonged boiling of these ingredients was avoided. Agar was placed to a depth of 3/4-inch in 1-gallon jars. When cool, two or three holes were made in the agar with 3/8-inch glass tubing. Folded paper toweling was placed along one side of the jar and 3 or 4 drops of instant yeast were added to the agar. Jars were covered with paper toweling and once the yeast had started, culture jars were inoculated with 25 to 50 fruit flies. After mating—with the production of eggs, larvae, pupae and emerging flies—adequate numbers of *Drosophila* became available for use in testing pesticides.

Testing procedures consisted of LD_{50} determination using normal and toxic feed samples. One-pint glass milk bottles were used as a testing chamber. One gram of finely homogenized corn was placed in the chamber. One ml hexane was used as a vehicle to add varying amounts of pesticide. This was placed directly over the corn sample and allowed to evaporate over night. A small cotton ball soaked in dilute grape juice was added and the chamber agitated. Fifty to 75 fruit flies were added and the milk cap applied. The flies were attracted to the grape juice for moisture and became exposed to the toxicant. Similar methods without the hexane were used to test normal and toxic pesticide samples. Transfer of flies to the toxic chamber from the rearing jar was aided by use of a metal funnel.

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[2] Needham, P. H. 1960. An investigation in the use of bioassay for pesticide residues in food stuff. The Analyst, 85: 792-809.

[3] Sperra, P. R. 1961. A direct feeding method of bioassay of insecticide in soil with *Drosophila*. Journal of Economic Entomology. 54(4): 822-824.

[4] Scores, N.E.A. and Lichenstein, E. P. 1967. The use of *Folsomia fimetaria* and *Drosophila melanogaster* test insects for the detection of insecticide residues. Journal of Economic Entomology. 60(6): 1539-1541.

[5] Sulphur-free molasses proved to be advantageous.

Results

The estimated time to kill 50 percent of the *Drosophila* in the presence of various insecticides, including a few herbicides and other chemical toxicants, was calculated from test results. These results are presented in table 1.[6]

Table 1.—Pesticides and ppm tested with Drosophila
for LD_{50} kill time

Pesticide	ppm	LD_{50} in hours
Chlorinated Hydrocarbons and related compounds:		
Aldrin	10	19.29
Aramite	100	77.35
Atrazine	50	51.04
BHC	100	42.13
Chlordane	10	27.83
Dieldrin	10	32.25
DDT	200	24.45
Dichlorathane ethane	100	32.07
Endrin	10	27.38
Heptachlor	10	25.42
Kelthane	50	53.42
Lindane	10	47.69
Methoxychlor	10	71.90
Ovex	200	No deaths[1]
Perthane	100	42.13
Rothane (DDD)............	200	65.27
Strobane	50	31.63
Toxaphene	10	56.34
Nitrated Hydrocarbon:		
Karathane	100	No deaths
Organic phosphate:		
Malathion	20	21.84
Phosdrin	20	1.59
Vapona (DDVP)	10	0.125
PCB's:		
Arachlor 1242	50	66.17
Arachlor 1555	20	46.41
Pyrethrum:		
Pyrethrum extract	100	70.28
Carbamate:		
Methyl carbamate	200	72.69

[1] No deaths were recorded for the experimental 72 hours.

From the data shown *Drosophila* responded rapidly to the organic phosphate Vapona (DDVP) with results for the chlorinated hydrocarbons varying with the compound used. The behavioral characteristics of the *Drosophila* exposed to the various chemical compounds indicated differences varying from muscular twitching, nervous spasms, arched wings, droopiness, inability to fly, short hopping movements, to few signs other than death itself.

Several raw feed samples sent to the laboratory for chemical analysis, when biotically tested, indicated either a positive or negative response.

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[6] Pesticide samples were prepared by the Toxicology Unit, Veterinary Services Diagnostic Laboratory, Ames, Iowa.

Discussion

Seventy-two hours was selected as the biotic screening time for testing *Drosophila*. In the absence of nutrition and toxicants, but with adequate moisture and 1 ml evaporated hexane, it was estimated by statistical analysis that only about 3.75 percent of the fruit flies would die within 72 hours. Thus, we are reasonably confident that in the great majority of cases, less than 10 percent of the *Drosophila* would die in the absence of toxicants within the test period enabling a 95 percent confidence limit.

In this report, a direct bio-screening method using *Drosophila* mortality as an indicator was described. The test was designed for use on feed samples suspected of containing chemical residues such as insecticides. It might also be applicable for stomach or rumen contents.

A positive test (fly mortality) would not identify the toxic substance but would indicate the need for chemical qualitative analysis.

The expense and difficulty of maintaining a colony of flies was negligible. The test described might be utilized at State and local laboratories, especially when other chemical testing facilities are not available.

Acknowledgments

SALMONELLOSIS

AN EVALUATION OF A FLUORESCENT ANTIBODY TECHNIQUE FOR DETECTING SALMONELLAE IN TISSUES FROM POULTRY. Harrington, R., Moulthrop, I. M., Stein, G., and Snedeker, C. J. (Project Report)

Summary

Comparisons were made between Selenite-F (SF) broth, gram negative (GN) broth, and a fluorescent antibody (FA) technique for the detection of salmonellae in tissues from poultry.

Of the 274 specimens examined, 101 (36.8 percent) were positive by FA, 95 (34.6 percent) were positive by SF broth culture, and 71 (25.9 percent) were positive by GN broth culture. The FA technique was more sensitive than cultural methods employed. The time required for the detection of salmonellae by FA was 18 to 20 hours whereas SF and GN broth culture required a minimum of 72 hours.

Introduction

Salmonellosis is one of the important bacterial diseases affecting all types of poultry.[1] Present cultural methods for the isolation of salmonellae require a minimum of 72 to 96 hours. A rapid laboratory diagnostic method would be desirable for detecting salmonellae in tissues from chickens and turkeys.

In a previous report, a fluorescent antibody (FA) technique was described for the detection of salmonellae in swine tissues.[2] The purpose of this study was to determine the efficiency of the FA technique when applied to tissues from poultry.

The results of studies comparing a FA procedure with two cultural methods are reported.

Materials and Methods

Source of tissues—Tissues were obtained from field cases submitted for bacteriologic examination to the Regional Diagnostic Laboratory, Salisbury, Md.

Conjugate—Salmonella conjugate was provided by the General Bacteriology Unit, Diagnostic Services, National Animal Disease Laboratory (NADL). Procedures for the preparation of the conjugate were described earlier.[3]

Media—SF broth,[4] GN broth,[5] triple sugar iron (TSI) agar[5] and lysine iron (LI) agar[5] were prepared according to the instructions of the manufacturer. Brilliant green sulfadiazine (BGS) agar was prepared according to methods described in ARS 91-68, revised May 1968.[6]

Culture Methods—Approximately 1 gram of tissue cut into small pieces was placed into a test tube containing 10 ml of SF broth. The same procedure was used with GN broth. After overnight incubation at 37° C., a 5 to 6 mm loopful of GN and SF broth cultures was streaked on BGS agar plates. Typical colonies appearing on BGS agar

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[1] Williams, J. E. Paratyphoid and Arizona infections. *Diseases of Poultry,* 5th ed. Edited by H. E. Biester and L. H. Schwarte. 260-328. Iowa State University Press, Ames, Iowa.

[2] Harrington, R., E. M. Ellis, E. T. Mallinson, M. Ranck, and R. E. Solee. An evaluation of a fluorescent antibody technique for the detection of salmonellae in animal by-products, Feeds, and Tissues. J.A.V.M.A. 157 (11): 1898-1900, 1970.

[3] Ellis, E. M. and R. Harrington. A direct fluorescent antibody test for salmonella. Application in examining animal feeds and by-products. Arch. Environ. Health 19: 876-881, 1969.

[4] Baltimore Biological Laboratory, Baltimore, Md.

[5] Difco Laboratories, Detroit, Mich.

[6] Recommended procedure for the isolation of salmonella organisms from animal feeds and feed ingredients. ARS 91-68, Agricultural Research Service, U. S. Department of Agriculture, May 1968.

following 24 to 48 hour incubation were selected and transferred to TSI and LI agar slants. Those cultures producing reactions characteristic of the salmonellae were tested with polyvalent "0" antiserum according to a standard procedure.[6]

FA technique- The fluorescent antibody (FA) procedures were those previously described.[2]

Results and Discussion

Laboratory examinations were made on 274 specimens for the detection of salmonellae (table 1). The data indicate that the FA technique was more sensitive than the culture methods. This is in agreement with results previously reported. [2] [3]

Table 1.– Comparison FA and cultural results

Method	Positive	Percent positive
FA	101	36.8
SF[1]	95	34.6
GN[2]	71	25.9

[1] Selenite-F broth.
[2] Gram negative broth.

A further breakdown of the results are summarized in tables 2 and 3. In comparisons between the FA technique and GN broth culture there was agreement on 231 of the 274 specimens examined. However, 35 were FA-positive/culture-negative and 5 were FA-negative/culture-positive. The amount of agreement between SF broth culture and the FA technique was the same as observed with FA and GN broth culture. Nevertheless, there was a decrease in the number of specimens found FA-positive/culture-negative and an increase in the number of specimens FA-negative/culture-positive.

Table 2. FA and GN[1] cultural results

Specimen	Number	FA-/GN-	FA+/GN+	FA+/GN-	FA-/GN+
Liver:					
Chicken.	262	164	61	35	5
Turkey	5	0	1	0	0
Pheasant	5	4	2	0	0
Brain (chicken)	2	0	2	0	0

[1] Gram negative broth.

Table 3.—F 1 and SF[1] culture results

Specimen	Number	FA-/SF-	FA+/SF+	FA+/SF-	FA-/SF+
Liver:					
Chicken	262	158	67	20	17
Turkey	5	0	3	2	0
Pheasant	5	4	0	1	0
Brain (chicken)	2	0	2	0	0

[1] Selenite-F broth.

From the results of this study it was concluded that the FA technique provided a rapid means for detecting salmonellae. However, since the FA slides were prepared from GN broth cultures only, and 8.7 percent more salmonella isolates were recovered from SF broth cultures, it would have been interesting to have applied salmonella conjugate to smears prepared from SF broth culture.

A fluorescent antibody technique (FAT) was used for the detection of salmonella in porcine tissues. The method was based on enrichment of tissues in gram-negative (GN) broth and then fluorescent antibody detection of salmonella cells. Tissues were also cultured in tetrathionate (TT) broth for comparison. Of the 317 specimens examined, 114 were test positive by FAT, 66 were test positive by culture in TT broth, and 64 were test positive by culture in GN broth. The FAT seemed to be a far more sensitive method than the cultural techniques. The FAT provided a rapid method for the detection of salmonellae in porcine tissues, reducing the time to 18 to 20 hours.

PATHOLOGIC AND HEMATOLOGIC CHANGES OBSERVED IN SWINE INOCULATED WITH SALMONELLA CHOLERAESUIS VAR. KUNZENDORF AND ITS ENDOTOXIN. Sherman, K. C.
Thesis submitted as partial fulfillment of Master's Degree, Iowa State University, Ames, Iowa, 1972. (Abstract of Thesis)

The objective of this study was to determine the role of endotoxin extracted from *Salmonella choleraesuis* var. *kunzendorf* in the clinical, hematologic, and pathologic manifestations of acute salmonellosis.

Live salmonellae, killed salmonellae and extracted endotoxin were inoculated into three different groups of pigs which were surgically prepared with indwelling vinyl catheters. The catheters were inserted into the femoral arteries and were necessary for the frequent blood collection regimen. Control pigs were catheterized and subjected to identical procedures as the experimental pigs.

Clinical similarities included elevated temperatures, vomiting, transient diarrhea followed by constipation, depression, spontaneous bleeding and profuse salivation. The effect of endotoxin was definitely pyrogenic. It also affected the parasympathetic side of the autonomic nervous system, and had a marked effect on the clotting ability of the blood. Large doses of endotoxin often resulted in shock.

The hematologic changes were studied and characterized. The injection of endotoxin resulted in increases in the erythrocyte sedimentation rates. The packed cell volume values and total red blood cell counts were not significantly altered. Serum enzyme studies revealed increased levels of activity of both serum glutamic oxaloacetic transaminase and lactose dehydrogenase. The main effects of endotoxin were on the total white blood cell counts and differential leukocyte counts. A severe leukopenia was produced in the early stages following injection with endotoxin. The total white blood cell counts of approximately 15,000 cells/cm of blood decreased to approximately 1,000 cells/cm by the 4th hour post inoculation. Differential leukocyte counts revealed segmented neutrophils to be almost totally driven from the main stream of the circulation. A leukocytosis was evident 24 hours post inoculation, with the total white blood cell count elevated 3 to 4 times pre-inoculation values. Immature cells of the neutrophilic granulocytic series predominated during the leukocytosis.

Lesions were similar in all pigs although each group received a different form of the inoculum. Characteristic "typhoid nodules" were found in the endotoxin injected pigs as well as in the ones that received live salmonellae. A diffuse reticuloendothelial cellular hyperplasia was a consistent finding in the spleens. The lungs had foci of cellular infiltrations associated with damaged alveolar capillaries. Some capillaries were actually occluded with neutrophils, lymphocytes and hypertrophied endothelial cells of the capillaries. The lung lesions persisted for some time after the pigs began to recover.

No lesions were noted in the central nervous system. No intestinal damage was demonstrated in the acute form of salmonellosis.

Only single doses of organisms and endotoxin were given to avoid the production of lesions associated with the generalized Schwartzman reaction, which would complicate the findings of simulated field cases of acute salmonellosis.

SALMONELLA SEROTYPES IDENTIFIED FROM ANIMAL SOURCES IN THE UNITED STATES
DURING FISCAL YEARS 1967-1972. Blackburn, Billie O. Paper given at the
U.S.-Japan Natural Resources Program, Mycoplasmosis Panel, Athens, Georgia,
December 5-6, 1972.

Summary. The accumulated statistics seem to indicate that there are some definite trends in the salmonella serotypes being identified. The trends go both directions—some serotypes are being identified more frequently and others less frequently.

Only three trends were examined to the fullest extent possible.

Salmonella san diego showed a definite trend to more frequent identification when compared to all other serotypes from all sources. The trend was especially evident in isolations from turkeys from the States of Illinois, Minnesota and Utah.

S. worthington showed somewhat of a trend to more frequent identification when the source was not specified. When individual sources were considered, the upward trend was very evident in cultures from chickens. The State of Georgia contributed most to this trend, but the Southeast in general followed the same pattern.

S. newport seemed to show a definite trend toward being more common when all sources were considered together. From specific sources the trend seems definite in cultures from swine and suggestive in cultures from cattle. Further studies of this trend were restricted due to the small numbers of isolations.

Introduction

Salmonella serotyping statistics are important to the epidemiologist who is engaged in the study of salmonellosis whether they involve animals or man. The purpose of this report is to provide some statistical information from the salmonella serotyping laboratories associated with Veterinary Services, APHIS, USDA.

Federal Laboratories

Ames, Iowa
Phoenix, Ariz.
Atlanta, Ga. (closed at the time of this report)

Other Laboratories

Orono, Me. - University Laboratory
Salt Lake City, Utah - State Dept. of Agriculture
Amherst, Mass. - University Laboratory
Madison, Wis. - State Dept. of Agriculture
Barron, Wis. - State Dept. of Agriculture

In 1967, data from the salmonella serotyping laboratories began to be stored by computer. The statistics in this report were retrieved from this source. All cultures, other than those associated with the Cooperative State-Federal Rendering Plant Program, are included. Thus, the statistics are somewhat crude.

These statistics will cover the period beginning with fiscal year 1967 through fiscal year 1972. The total numbers of salmonella cultures serotyped by fiscal year are as follows:

Fiscal year	Cultures serotyped
1967	8,436
1968	9,859
1969	10,650
1970	11,496
1971	6,853
1972	8,656

The sharp drop in cultures serotyped in 1971 could be misleading. The drop was most likely due to a concerted effort to reduce the serotyping work load. Isolating laboratories were encouraged to use more serologic tests (common O Groups [B, C_1, C_2, D, E_1, E_2, E_4] and Spicer-Edwards 11 tests) and to be more selective when sending cultures for serotyping.

The increase again in 1972 probably reflects the fact that Salmonellae continue to appear more frequently in isolating laboratories.

A popular approach to salmonella statistics is to study the relative prevalence of the different serotypes. This information is provided in table 1. Serotypes ranked 1 through 6 in 1967 remain rather high in rank throughout the 6 years. Serotypes showing a trend to lower rank include Montevideo, Schwarzengrund, Einsbuettel, Blockley and Chester. Serotypes showing a trend to higher rank include Worthington, Choleraesuis var. Kunzendorf, San diego and Newport.

Table 1.—Common Salmonella serotypes ranked by fiscal year

Serotype	Fiscal year					
	1967	1968	1969	1970	1971	1972
Heidelberg	1	1	1	1	2	3
Typhimurium	2	2	4	2	1	2
Typhimurium var. Cop.	3	3	12	12	7	5
Infantis	4	7	9	11	8	8
Saint paul	5	5	8	5	3	4
Anatum	6	4	5	3	4	7
Montevideo	7	6	2	7	11	14
Schwarzengrund	8	18	25	25	17	15
Eimsbuettel	9	8	6	8	19	16
Senftenberg	10	14	3	6	6	11
Blockley	11	12	19	17	10	22
Thompson	12	9	15	10	16	12
Worthington	13	27	20	9	9	10
Chester	14	25	43	30	30	29
Derby	15	17	13	15	21	20
Cholerae-suis var. Kunz.	16	11	7	4	5	6
San diego	17	21	29	16	13	1
Panama	18	44	53	50	57	50
Tennessee	19	15	16	13	12	25
Bredeney	20	22	10	14	22	17
Binza	21	24	14	32	24	21
Newport	22	32	31	23	14	9

Information on serotypes identified from specific sources is also of value. Tables 2 through 5 give the rank of common serotypes from turkeys, chickens, swine and cattle respectively for the period covered. Trends as indicated in the tables may be summarized as follows:

Serotypes Showing Trends in Turkeys

Down trend	*Up trend*
Schwarzengrund	San diego
Chester	Senftenberg
Panama	Reading

Serotypes Showing Trends in Chickens

Down trend	Up trend
Heidelberg	Worthington
Enteritidis	
Pullorum	

Serotypes Showing Trends in Swine

Down trend	Up trend
Heidelberg	Newport
Anatum	

No specific trends are evident from the table on serotypes from cattle. Also, the total number of cultures involved from cattle was much less than from the other sources.

A comparison of the serotypes from different sources showing down trends is as follows:

All sources	Turkeys	Chickens	Swine
Montevideo			
Schwarzengrund	Schwarzengrund		
Eimsbuettel			
Blockley			
Chester	Chester		
Panama	Panama		
		Heidelberg	Heidelberg
		Enteritidis	
		Pullorum	
			Anatum

A comparison of the serotypes from different sources showing up trends is as follows:

All sources	Turkeys	Chickens	Swine
San diego	San diego	— —	—
Worthington	— —	Worthington	— —
Choleraesuis var. kunz.	—	—	— —
Newport	—	— —	Newport
—	Senftenberg	— —	—
—	Reading	— —	—

Table 2. Salmonella serotypes from turkeys ranked by fiscal year

Serotype	Fiscal year					
	1967	1968	1969	1970	1971	1972
Heidelberg	1	1	1	1	1	2[a]
Saint paul	2	2	2	2	2	2[a]
Schwarzengrund	3	6	_[b]	–	8	8
Chester	4	8	–	9	–	–
Anatum	5	5	4	4	7	5
Typhimurium	6	3	3	3	4	7
San diego	7	4	5	6	3	1
Panama	8	–	–	–	–	–
Senftenberg	9	–	6	5	5	6
Typhimurium var. Cop.	10	–	–	–	–	–
Reading	–	9	–	10	6	4

[a] Equal rank.
[b] - Indicates a rank below 10.

Table 3.—Salmonella serotypes from chickens ranked by fiscal year

Serotype	Fiscal year					
	1967	1968	1969	1970	1971	1972
Heidelberg	1	1	1	1	6	7
Infantis	2	4	6	6	2	1
Typhimurium var. Cop.	3	2	3	8	3	4
Typhimurium	4	5	4	6	3	5
Thompson	5	3	2	2	7	3
Montivedeo	6	9	9	9	8	8
Blockley	7	6	5	5	1	10
Saint paul	8	8	7	10	–[1]	9
Enteritidis	9	7	8	3	–	–
Pullorum	9	10	10	–	–	–
Worthington	–	–	–	4	5	2

[1] Indicates a rank below 10.

Table 4.—Salmonella serotypes from swine ranked by fiscal year

Serotype	Fiscal year					
	1967	1968	1969	1970	1971	1972
Cholerae-suis var. Kunz.	1	1	1	1	1	1
Typhimurium	2	2	2	2	2	2
Derby	3	5	3	3	3	3
Heidelberg	4	4	6	4	5	8
Anatum	5	7	4	5	10	9
Saint paul	6	9	–[1]	10	4	4
Typhimurium var. Cop.	7	3	5	6	7	7
Infantis	7	–	8	7	10	4
Enteritidis	–	6	7	–	10	9
Newport	–	–	9	8	8	4

[1] Indicates a rank below 10.

Table 5.—Salmonella serotypes from cattle ranked by fiscal year

Serotype	Fiscal year					
	1967	1968	1969	1970	1971	1972
Typhimurium	1	1	1	1	1	1
Typhimurium var. Cop.	2	2	2	2	3	3
Dublin	3	3	6	3	5	4
Newport	4	4	3	4	2	2
Saint paul	5	–[1]	4	5	8	8
Anatum	6	–	5	7	6	7
Heidelberg	7	–	–	6	4	5
Infantis	7	–	–	8	–	–
Enteritidis	–	4	8	–	–
Thomasville	–	6	–	–	–	–

[1] Indicates a rank below 10.

The information which has been accumulated permits a more detailed study. Howe er, in this report, furthe study will be limited to San diego from turkeys, Worthington from chickens and Newport from swine. Tables 6, 7 and 8 have been prepared to show the States where the isolations were made.

Table 6.—San diego identifications from turkeys

State	Fiscal year					
	1967	1968	1969	1970	1971	1972
Arkansas	1	0	4	4	4	1
California	16	5	0	1	1	35
Illinois	3	0	0	0	1	61
Minnesota	7	5	30	23	64	47
Missouri	0	0	5	1	1	6
Nebraska	0	0	0	1	1	3
North Carolina	2	3	0	5	3	2
Oregon	12	12	9	10	11	1
South Dakota	2	0	1	5	5	4
Texas	15	23	6	2	0	8
Utah	2	10	26	11	50	574
All others	22	6	3	10	6	5
Total	82	64	84	73	147	747

Table 7.— Worthington identifications from chickens

State	Fiscal year					
	1967	1968	1969	1970	1971	1972
Alabama	0	0	0	19	16	8
Florida	1	1	1	2	5	0
Georgia	2	4	2	44	63	125
Idaho	1	10	18	9	0	0
Illinois	0	0	0	0	1	7
Indiana	0	0	0	0	4	4
Maryland	0	0	3	4	6	0
Mississippi	2	0	0	3	1	7
North Carolina	0	0	1	17	16	1
Ohio	0	0	0	4	0	1
Tennessee	0	0	0	18	4	6
All others	10	5	2	3	4	7
Total	16	20	27	123	120	166

Table 8. –Newport identifications from swine

State	Fiscal year					
	1967	1968	1969	1970	1971	1972
Arkansas	1	0	2	2	0	0
Florida	0	0	2	0	0	0
Illinois	0	0	0	1	1	1
Indiana	0	0	0	1	2	9
Iowa	0	0	0	0	1	0
Kentucky	0	0	1	2	1	0
Louisiana	0	0	0	1	0	0
Maryland	0	0	0	1	1	1
Minnesota	0	0	0	1	0	4
Mississippi	1	0	1	0	0	0
Missouri	0	0	0	0	0	2
Nebraska	0	0	0	2	0	0
New Mexico	0	0	1	0	0	0
South Carolina	0	0	0	1	2	1
South Dakota	0	0	0	0	1	0
Tennessee	0	1	0	1	0	1
Texas	0	0	3	5	0	0
Unknown	0	2	0	0	0	0
Total	2	3	10	18	9	19

SCRAPIE

SCRAPIE DIAGNOSIS: CORRELATION OF HISTOPATHOLOGY AND MOUSE INOCULATION
STUDIES. *Miller, L. D., Jenkins, S. J., Sherman, K. C., Gigstad, D. C.,
Muhm, R. L. and Klingsporn, A. L. (Project Report)*

Summary

Sections of brain tissue from 12 sheep and 3 goats were examined microscopically for lesions of scrapie. Portions of each were inoculated into susceptible mice as an in vivo test for scrapie. The results were comparable in 10 cases. Five cases, in which a histopathologic diagnosis of scrapie was not possible, were found to harbor the agent by in vivo testing (mouse inoculation). Two of these five animals died with intercurrent disease and post mortem autolysis precluded adequate microscopic examination in two others.

These studies illustrate the importance of proper specimen preservation and the necessity of mouse inoculation when a clinical diagnosis of scrapie cannot be confirmed by histopathologic examination.

Introduction

Scrapie is a natural disease of sheep and was first reported in the United States in 1947 but has been known in England, Germany and France for more than 200 years.[1]

The onset of clinical disease is insidious. Initial signs are increased nervousness and excitability which slowly develop into incoordination and tremors. The course is relentlessly progressive and death may occur in a few days or after several months. An assortment of other clinical signs attributable to a central neurological disturbance may appear including pruritis, ataxia and nibbling movements of the lips. Extensive wool loss from constant rubbing and emaciation often occur late in the course of disease. The intensity of clinical signs may vary considerably. There are no characteristic gross lesions but an increase in the volume of cerebrospinal fluid is not uncommon.

In some animals that die soon after clinical signs appear, histologic changes may be poorly developed. Those animals with a more extended course usually develop the expected central nervous system lesions. These lesions are quite specific and often the severity is increased in animals with a prolonged clinical course.

In 1961, transmission of scrapie to laboratory mice was reported[2] and since then mouse inoculation has become an integral part of most diagnostic and investigational work. Goats, rats, gerbils, mink and monkeys have also been infected experimentally.[1]

A project was initiated to compare histopathologic findings with the results of mouse inoculation studies.

Several sheep or goats provided brain tissue for histologic examination and for mouse inoculation. The donor animals were clinical cases of scrapie, progeny of sheep from infected bloodlines or otherwise suspected of harboring the scrapie agent.

Materials and Methods

Brain tissue was obtained from 15 sheep and goats from the APHIS Scrapie Field Trial, Mission, Tex.

A 10 percent suspension of finely ground brain tissue was prepared in 0.85 percent saline containing 10 percent rabbit serum and antibiotics. The material was centrifuged at 2,500 RPM for 30 minutes to remove the larger particles. The supernate was decanted and 0.02 cc was inoculated intracerebrally into weanling mice. At least 20 mice were inoculated with each preparation.

A 2-year observation period followed. Mice that died during the first 2 months were excluded from the study.

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[1] Lampert, P. W., Gajdusek, D. C., and Gibbs, C. J.: Subacute spongform virus encephalopathies. Amer. J. Path. 68:626-652, 1972.

[2] Chandler, R. L.: Encephalopathy in mice produced by inoculation with scrapie brain material. Lancet 1:1378-1379, 1961.

Brain tissues from donors and recipients were preserved in neutral buffered saline with 10 percent formalin. Sections were prepared for microscopic examination by routine histological procedures and by Cajal's method.

Results

The microscopic lesions expected in scrapie were observed in five of the donor animals (table 1). Post mortem decomposition precluded proper microscopic evaluation in two cases and scrapie lesions were not observed in the remaining eight. Intercurrent disease accounted for the death of five animals, two of which had scrapie lesions.

Signs and lesions of scrapie were observed in 10 of the 15 groups of inoculated mice (table 1). The donors for the positive groups were the five with lesions of scrapie, two sheep with autolytic changes, two sheep that succumbed with intercurrent disease in which scrapie lesions were not observed, and one sheep showing no signs of scrapie which was sacrificed at 93 months of age.

Table 1.—Histopathology of donor animals and reactions of inoculated mice

Donor	Species	Age in months	Scrapie lesions	Scrapie in inoculated mice
G-30	Sheep	43	? PM Autolysis	+
G-158	---do---	33	+	+
G-254	---do---	33	? PM Autolysis	+
JD-18[1]	---do---	36	−	+
G-205	---do---	96	−	−
G-05[1]	---do---	69	−	−
G-209	---do---	72	−	−
G-68	---do---	33	+	+
G-119	---do---	93	−	+
G-20[1]	---do---	42	−	+
G-211	---do---	104	−	−
G-133	---do---	8	−	−
B-261[1]	Goat	43	+	+
B-259[1]	---do---	44	+	+
B-258	---do---	45	+	+

[1] Died or sacrificed due to intercurrent disease.

Discussion

In sheep with the well developed scrapie lesions of neuronal degeneration including vacuolation, gliosis or more specifically astrocytosis and spongiform degeneration of the intercellular ground substance, an accurate histopathologic diagnosis can be made. Mouse inoculation may be essentially a confirmatory test in such cases.

Mouse inoculation studies assume greater significance when histopathology is uncertain, as in the presence of advanced autolysis or when lesions are not sufficiently developed to be conclusive.

Scrapie lesions were not observed in three sheep (JD-18, G-119, G-20), however, inoculated mice did develop the disease. Natural scrapie occurs most often in sheep ranging in age from 35 to more than 50 months[3]; and sheep JD-18 and G-20 may have developed diagnostic lesions had they not died with intercurrent disease. Sheep G-119 was 93 months of age when sacrificed, yet showed no clinical signs of scrapie. An important epidemiological question is whether such animals shed the scrapie agent as well as harbor it. Scrapie has been reported in the progeny of some bloodline sheep that were apparently refractory to the disease (3), but their role in transmission, if any, is not certain.

[3]Klingsporn, A. L. and Hourrigan, J. L.: Spread of scrapie among sheep and goats. Proc. 75th Ann. Mtg. USAHA, October, 1971.

SWINE ERYSIPELAS

A FLUORESCENT ANTIBODY TEST FOR ERYSIPELOTHRIX RHUSIOPATHIAE.
Hulse, Donald C. (Project Report)

Summary

A fluorescent antibody (FA) technique has been developed for the detection of *Erysipelothrix rhusiopathiae*. There was approximately 97 percent agreement between the FA and biochemical tests for the identification of this organism. The FA technique provided a rapid method for detecting *E. rhusiopathiae*.

Introduction

Erysipelothrix rhusiopathiae (2),[1] or *E. insidiosa* (1), was first isolated by Robert Koch (4) from a septicemic mouse in 1878. Pasteur (7) briefly described a similar organism isolated from swine in 1882. *E. rhusiopathiae* was first isolated from the kidney of a pig in the United States in 1885 (10).

Erysipelothrix rhusiopathiae can cause polyarthritis in sheep, and is of major economic importance to the turkey and swine industries (9). From 1951 to 1960, the average annual loss in swine due to erysipelas in this country was approximately $5.5 million (11). The clinical signs of swine erysipelas are characterized by septicemia, often accompanied by pathognomonic rhomboid uticarious lesions, commonly referred to as "diamond skin" lesions. This organism may produce lesions and clinical signs indistinguishable from hog cholera.

Erysipelothrix rhusiopathiae has long been recognized as being pathogenic for man and animals. The development of a rapid detection method for this organism would, therefore, be desirable in a diagnostic laboratory. Marshall, *et al.* (6) were the first to develop a fluorescent antibody (FA) test for *E. rhusiopathiae;* however, a limited number of cultures was used in the investigation.

The purpose of this study was to develop an FA technique for the detection of *E. rhusiopathiae*.

Materials and Methods

Antisera.—*Erysipelothrix* antisera from two different species of animals (rabbits and horses) were used in preparing conjugates. The culture (M_3Lp_3) used to prepare the rabbit antiserum was furnished by Dr. R. D. Shuman, North Central Region ARS, NADL. The rabbit antiserum (8) was fractionated with 50 percent ammonium sulfate and labeled with fluorescein siothiocyanate (25 micrograms dye/milligram protein). Horse antiserum was obtained from Anchor Serum Co. (Division of Philips Roxane). The horse antiserum was precipitated with half-saturated ammonium sulfate and redissolved in cold distilled water. The globulin fraction was then dialyzed at 4° C. with physiologic saline to remove the ammonium sulfate. Phosphate buffered saline (pH 9), 0.2 M, was used to buffer the globulin which was conjugated by adding fluorescein isothiocyanate (25 micrograms dye/milligram protein) for 5 hours at 25° C. The conjugated globulin was then dialyzed in 0.01 M phosphate-buffered saline (pH 7.6) at 4° C. to remove excess dye. Primary studies indicated that the conjugate prepared from horse antiserum had a higher titer than that prepared from rabbit antiserum.

Cultures.—Cultures for comparison of the FA technique and biochemical tests were obtained from swine tissues submitted to the General Bacteriology Unit, Veterinary Services Diagnostic Laboratory, NADL, for bacteriological examination. The tissues were submitted by State and animal health programs regulatory veterinarians. Methods used for isolation and identification of *E. rhusiopathiae* have been described (3,12,13).

Using the conjugate prepared from horse antiserum, 323 cultures, morphologically and biochemically identified as *E. rhusiopathiae*, and 100 nonerysipelothrix cultures were examined by the FA technique. Each culture

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[1] Numbers in parenthesis refer to References at end of this paper.

was coded and transferred to a heart infusion agar slant and incubated for 18-24 hours. A bacterial suspension was prepared using 0.3 to 0.5 ml of physiological saline.

Smears. Smears were prepared by placing a 4 m loopful of the cell suspension on a clean non-fluorescing glass slide. The smears were air dried and fixed in Kirkpatrick's FA fixing solution (5). A drop of conjugate was applied to the smear which was then incubated in a 37° C moist chamber for 30 minutes. The slides were washed by passing them through three 10-minute changes of phosphate buffered saline (ph 7.6). The smears were mounted using FA mounting fluid.

Microscopy A Leitz Ortholux microscope with a BG 12 blue-excitor filter and a K 530 barrier filter was used to view fluorescence. The light source was a high intensity xenon XBO 150W lamp. *E. rhusiopathiae* fluoresced a yellow-green color against a dark field.

Results

Fluorescent antibody examinations were conducted on 423 cultures (table 1). Of this number 312 were positive and 111 were negative. Eleven cultures biochemically identified as *E. rhusiopathiae* were negative by the FA technique. In addition to the biochemically positive cultures, 100 nonerysipelothrix cultures were also examined to determine specificity of the conjugate. None were FA positive.

This study indicated there was approximately 97 percent agreement between the FA technique and biochemical tests for the identification of *E. rhusiopathiae*. Biochemical identification of *E. rhusiopathiae* in pure cultures requires 48 to 72 hours while FA detection requires only 18 to 24 hours. Therefore, the FA technique would facilitate more rapid laboratory diagnoses of swine erysipelas and erysipelothrix infections in man and other animals.

Table 1.—The biochemical and FA results on 423 *Erysipelothrix* and non-*Erysipelothrix* cultures

		FA results	
Biochemical results		Positive	Negative
Positive 323		312	11
Negative 100		--	100

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TICKS

During calendar year 1971 the collection and submission of ticks from native and imported animals were 40 percent greater than for 1970. There were 4,247 species collections in 1971, compared with 3,025 in 1970.

The tropical bont tick, *Amblyomma variegatum* was found on a sheep at an abattoir on St. Croix, U. S. Virgin Islands, in March 1971. Subsequently tropical bont ticks were found on sheep, cattle, and goats at two farms in addition to the farm from which the sheep at the abattoir had originated.

In 1971, a serious outbreak of cattle fever ticks (*Boophilus* spp.) occurred in Cameron County, the most southerly county in Texas, adjacent to the Mexican border. Cattle fever ticks were found on 31 premises in Cameron County. As a result of the Cameron County outbreak, a total of 88 premises in 11 counties in Texas were infested or exposed.

The fowl tick, *Argas persicus*, was found on chickens in Maryland and Pennsylvania in 1971. The Maryland infestation is especially interesting because of the unusually high death losses in the small farm flock. The owner reported that he lost 85 percent of 100 young pullets within 1 month after they were placed in the very heavily infested poultry house. All clinical signs indicate that the birds died of tick paralysis.

VESICULAR STOMATITIS

*APPLICATION OF FLUORESCENT ANTIBODY TECHNIQUE FOR IDENTIFICATION OF
VESICULAR STOMATITIS VIRUS ISOLATED FROM FIELD SPECIMENS. Brown, C. L.[1]
Jenney, E. W., and Senne, D. A. (Project Report)*

Summary

Fifty-three vesicular stomatitis (VS) virus isolates were obtained from 102 specimens by the fluorescent antibody cell culture technique (FACCT). The specimens were mostly bovine epithelium received during VS outbreaks. At least 98 percent agreement was obtained on typing these isolates by the fluorescent antibody (FA) test compared with the complement-fixation (CF) and/or the virus isolation (VI) and neutralization techniques.

Introduction

The FACCT for VS has been described.[2][3] The purpose of this study was to compare FACCT methods with conventional methods with respect to speed and accuracy for the diagnosis of VS. These methods included CF and VI. The neutralization test using typing antiserums required 2 to 3 days to identify virus isolates; the FA method requires only the application of fluorescent labeled antibody to virus infected tissue culture (TC) as soon as the cytopathic effect (CPE) is observed, usually by 24 hours.

Initial plans were to test vesicular specimens as they were received at the laboratory. Since the incidence of clinical VS was so low as to preclude an adequate sampling during the study period, it was decided to retest samples that had been collected during earlier outbreaks and preserved at -85° C.

Materials and Methods

Ninety-five specimens of epithelium were each ground by mortar and pestle and 20 percent suspensions were prepared in phosphate buffered saline. Suspensions were centrifuged and an antibiotic mixture of streptomycin, penicillin, achromycin and amphotericin B[4] was added. Tenfold dilutions of each specimen were prepared and four Leighton tissue culture tubes containing coverslips were inoculated with the 10^0 - 10^{-3} dilutions. Monolayer cultures of embryonic bovine kidney (EBK) or embryonic swine kidney (ESK) in Earle's medium containing 5 percent SPF calf serum were used as the host system. One tenth ml of the virus suspension was placed in each tube and cultures were held at 35° C. When CPE was detected in the cultures, one coverslip each was stained with New Jersey (NJ) and Indiana (Ind) type conjugates. Six sets of coverslips that did not have CPE after inoculation were stained for negative controls.

In addition to the epithelial tissue, vesicular fluid, two TC isolates and five probang samples were also tested. The probang samples were treated with a fluorocarbon, trichlorotrifluorethane (TTE), according to Sutmoller and Cottral[5] and inoculated into Falcon flasks containing ESK. When CPE was observed, a transfer was made to Leighton tubes for the FA test.

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[1] Present address: 1523 Hauser, Helena, Mont. 59601.

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The techniques for virus isolation, CF[6] and neutralization[7] were previously described. Results of the FACCT from each of the samples of epithelium were compared to the original complement-fixation and virus isolation results.

Results

Ninety-five specimens including one vesicular fluid, two TC isolates and five probang samples were tested. Forty-six NJ virus isolates and seven of the Ind type were identified by FACCT. In all, except two of these 53 specimens, FA stains were made simultaneously with conjugates of NJ and Ind VS types.

There were six specimens from which VS virus was isolated before storage that were negative when examined by the FACCT. In three cases virus was not isolated by routine virus culture but was detected by the FACCT.

Results with one sample were unexplainable. Originally, this specimen was diagnosed as NJ VS with the CF test; however, the Ind type was detected with the FACCT. This would have been the first Ind VS virus isolated from Alabama. It was collected during an extensive outbreak of the NJ type involving Alabama and Georgia. It was considered most likely that this sample was mislabeled when other specimens were processed the same day with Ind type specimens.

Four other virus isolates were found in specimens negative for VS by the FACCT. Two were identified as bovine virus diarrhea virus by FACCT and the other two were not identified.

It was observed in this study that an interference phenomenon occurred in the test system. In 40 of 45 cases where virus was detected while using the dilution method, virus was recovered only at higher dilutions of 10^{-2} and 10^{-3}. Cytopathogenic effects were sometimes absent or slight in the undiluted 20 percent suspensions and the 10^{-1} dilutions. In several cases where the undiluted tubes were negative for virus, subcultures were performed without success.

Discussion

There was agreement of the conventional method of CF and VI neutralization with the FA method as to the type of VS virus detected except in the one case mentioned. Human error was more likely involved rather than failure of the FA test. Specimen suspension from this case was depleted, so repeat tests could not be performed. There was slightly over 98 percent agreement between the FACCT and the combined CF, VI, and neutralization techniques. The 95 percent confidence limit was calculated and found to be 91.5 percent; it was considered that there was at least 91.5 percent agreement between the two methods.

Two of the three positive probang specimens were taken 48 to 72 hours following intradermallingual inoculation and the other was a diagnostic specimen. In all three cases the esophageal-pharyngeal probang specimens were collected immediately following the harvest of unruptured tongue vesicles. Virus-laden saliva would obviously be present immediately following rupture of tongue vesicles so it was doubtful that the source of the virus was the esophageal epithelium. During a vesicular disease investigation, probang samples are taken when epithelium is not available.

Considering accuracy, ease and simplicity of the FA method, results of this study would support its use for the diagnosis of vesicular stomatitis.

Acknowledgments

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VIRUS DISEASES

The application of a potent antiviral conjugate is recommended to the diagnostic virologist provided he uses negative and positive controls. If a cell culture method is used the cells must be susceptible to the virus. This may require facilities for proper storage of frozen cell lines and a high level of competency in tissue culture propagation. If a tissue from the infected animal is sectioned, then skill and attention-to-detail are needed in this area. Control sections from all tissues should be stained with normal conjugate or serum-conjugate mixtures.

Reference virus strains should be used whenever possible and a bank of reference antiserums should be developed and maintained. Future work should be directed towards the production of new and better conjugates and the development of more precise systems for their utilization.

LABORATORY ASSISTANCE

LABORATORY ASSISTANCE TO THE PRACTITIONER; LABORATORY TEST RESULTS.
Carbrey, E. A. Paper given at the Arkansas Veterinary Medical Association
Meeting, Little Rock, Ark., February 11-13 1973.

Introduction

Tremendous advances have been made in the effective practice of both human and animal medicine in the last 30 years. However, in the care and treatment of livestock, as contrasted with pet medicine, certain logistic problems seem to remain unsolvable. The veterinarian must get in his specialized panel truck full of the latest gear and travel to the herd. On arrival, after the usual procedure of obtaining a history and doing the clinical and necropsy examinations, he is usually able to perform some simple laboratory procedures. He may even have a microscope and be able to confirm certain parasitic infections on the spot. The next decision and it is usually the most important one made on the case is to develop a tentative diagnosis. The next two steps should be simultaneous: First, he will prescribe advice and treatment; and second, collect suitable specimens for the laboratory examination that will confirm or reject his diagnosis.

How much more convenient it is for the physician or the veterinarian in pet practice with their patients in a hospital where all sorts of laboratory examinations can be performed. No such luxury for the animal industry veterinarian; he must select specimens with judgment, preserve them properly, request the appropriate examinations, and be able to understand and make effective use of the laboratory findings. It is the purpose of this presentation to provide some information on the effective use of the laboratory and how to interpret the results of some common diagnostic tests and procedures.

Ground Rules

There are certain basic considerations involved in specimen examination in the laboratory that must be spelled out if the game of "we-throw-the-stuff-in and you-throw-out-the-results" is to be mutually satisfactory. The need for adequate specimens is obvious—at least 5 to 10 ml of serum and 30 to 40 grams of tissue. Prompt preservation is important whether tissues are placed in formalin or refrigerated for agent isolation. Some of our most effective hog cholera diagnosticians pick up some dry ice on their way out to the suspected herd. All specimens should be carefully identified and the history, description and disease suspected or examination requested should be entered on a suitable submission form. The method of shipment to the laboratory will be directly related to the method of preservation. If an insulated polystyrene container is used, a generous amount of dry ice will keep the contents frozen for up to 96 hours while cans of frozen water will last only 24 hours. For short distances surface mail or air mail is adequate; but for distances of over 100 miles, air mail special delivery, air express, or air freight is faster and more reliable.

Agent Isolation and Identification

There would seem to be little need for discussing the isolation and identification of a pathogenic agent by the laboratory. The presence of such pathogens as *Bacillus anthracis*, *Mycobacterium bovis*, foot and mouth disease virus, or hog cholera virus in a specimen is prima facie evidence for the cause of the disease back on the farm. However, there are many borderline pathogens that may be isolated by routine cultural procedures. The isolation of an enterovirus from the intestinal tract is of little significance whereas the presence of the same virus in brain or spleen tissue is proof that this agent is an invasive pathogen. The enteroviruses may be classified into many serotypes by immunogenic or serologic characteristics. Certain serotypes such as the swine enterovirus ECPO 6 (Enteric Cytopathogenic Porcine Orphan) are considered more virulent due to their association with baby pig losses and clinical signs of central nervous system inflammation. If the laboratory identifies the isolate as to serotype, this may provide more specific information on pathogenicity to the clinician.

The same situation applies to bacteria. A salmonella obtained from swine tissue may create suspicion; but if the isolate can be identified as *Salmonella choleraesuis*, the laboratory has struck pay dirt. On several cases *S. choleraesuis* was isolated from the spleen while other *Salmonella spp.* were isolated from the gut. Although all *Salmonella spp.* are potential pathogens, *Escherichia coli* isolates must be serotyped to even furnish presumptive evidence of disease causing capabilities. Certain serotypes of *E. coli* have been associated with gastrointestinal disease but this was not a consistent finding for any particular serotype.

The presence of friendly and not so friendly bacterial denizens of the animal body is a well known fact to the general public. However, the intracellular parasitism of healthy animals by viruses is a surprise to many. Modern cell culture techniques have permitted the isolation and detection of viruses that, for want of a better name, have been designated "orphan" viruses. Such a virus is regarded as an agent in search of a disease. The confusing situation that may arise for the clinician is that the laboratory may isolate a virus that just happens to be there in the host's cells but is not responsible for the pathology observed.

Isolation and identification of a pathogenic agent usually implies a partial fulfillment of Koch's postulates. First, the agent grows as colonies or produces some effect such as dead embryos or cell destruction. Second, a specific typing antiserum is used to neutralize this effect and agglutinate or affect the agent in some easily recognized way.

There are other more rapid laboratory techniques used to detect the antigens of the agent by some serologic technique such as complement fixation, fluorescent antibody tissue section, and immunodiffusion. These techniques may be subject to false positive findings unless all the steps of the procedure are carefully controlled and the technician has considerable experience in reading the test.

Detection of Antibodies

The detection of the antibody response produced by an infectious agent is often of more interest to the epidemiologist than the practitioner. Usually the veterinarian is called to a scene of acute illness and unless the agent or its antigens can be quickly confirmed by the laboratory, he must wait for 10 to 20 days for a detectable antibody response to develop in the survivors. With some of the chronic diseases, such as brucellosis or EIA, the antibody titer is of much greater value since it is an indication of persistent infection. However, the conscientious veterinarian feels a responsibility to try to solve the diagnostic problem even if he must do so in retrospect by serologic evidence. There may be corrective measures that the farmer may wish to use in the future such as vaccination or sanitation. In addition, the veterinarian's diagnostic skill will be sharpened and the clinical profile of the specific disease will become obvious to him.

Serums should be collected at the initial visit and stored in the clinician's freezer until the second or convalescent set of serums is obtained from the same animals. These paired serums should be sent to the laboratory together. This may seem an imposition but there will be fewer mistakes if the bookkeeping is performed at the local level. With some diseases such as hog cholera, paired serums are less important since any significant titer is evidence for the past presence of the virus and a cause for concern to the veterinarian.

The reporting of serologic tests to the practitioner by the laboratory is probably the area where communication is least effective. Most serologic tests involve the titration or dilution of the serum and the determination of an antibody titer. An exception is the immuno-diffusion test used for EIA or bluetongue where only a positive or negative result is obtained. The serums are tested in a system where the presence of the specific antibody neutralizes the virus, fixes complement or inhibits hemagglutination. In many of these systems a serum obtained from a normal animal that has never been exposed to the disease agent will produce a positive reaction if straight or only slightly diluted serum is used. This low level of antibody is arbitrarily considered "nonspecific" although it may be due to cross reactions from infection with serologically related agents.

When using such test systems the serums must be diluted past this nonspecific titer and a significant titer must be determined at which the serum is considered positive. An example is the designation of the 1 to 25 titer for brucellosis as nonspecific, the 1 to 50 titer as suspect and the 1 to 100 as reactor or positive. A convenient rule is to regard the two lowest dilutions of the test to be in the suspicious category and consider as positive only those serums that have reactions in the third and higher dilutions. An example is the bluetongue complement fixation test in which the serum dilutions are 1 to 5, 1 to 10, 1 to 20, 1 to 40, etc. Serums that fix complement in the 1 to 5 or 1 to 10 dilution are considered suspicious while any serum fixing more than 50 percent of the complement in the 1 to 20 dilution is considered positive.

From the above discussion it is obvious that when the veterinarian receives a negative report on a serologic test from a laboratory he should also be told what dilution or titer of the serum was found negative. Some laboratories report this in a footnote, for example: "Negative serums produced less than 90 percent reduction of the virus plaque count at the 1 to 4 dilution." Another way to report the same finding is by the use of the symbol, "<" for "less than." For example with the above serum, "<" means there was less than the required degree of reaction at this dilution.

In evaluating the results of testing paired serums, a similar rule of thumb must be used in determining where a significant rise in titer has occurred between the two bleedings of a given animal. All serologic tests have a standard error of at least plus or minus one dilution. For instance, if a serum with a 1-16 Nt titer against infectious bovine rhinotracheitis was tested repeatedly, the titer would vary from 1 to 8 to 1 to 32. In other words, the standard deviation of this serologic test is plus or minus a twofold dilution. The application of this variable is important when one is deciding whether valid differences have been found in the titers of a group of paired serums. If the laboratory tested the same group of serums on different occasions, a certain number of serums would have higher or lower titers on the repeat tests. It is a good rule to have at least a two or more dilution increase in titer between the acute and convalescent bleedings before the rise is considered evidence for recent infection. In cases where the correct test is applied there will be some animals whose acute serums are negative while the convalescent serums have quite high titers. There will also be some animals in the same group that will show little or no change in titer.

The laboratory should report the serologic titers found and furnish an appropriate interpretation of these results. If a brief history is available and the serums properly identified, the interpretation will be more accurate.

Another source of confusion for the veterinarian is the way in which antibody titers or endpoints are expressed. Neutralization test endpoint titers are often reported as logarithms to the base 10. For instance:

Dilution	Log_{10}
1 to 10	1.0
1 to 100	2.0
1 to 1000	3.0
1 to 20	1.3
1 to 256	2.4
1 to 4	0.6
1 to 16	1.2

This may seem confusing until one realizes that the log_{10} titers increase as the dilution increases in simple arithmetic steps. When a 50 percent endpoint is calculated, it is easier to work with log_{10} titers than the actual dilution numbers. It is helpful to remember that the logarithms are exponents of 10 and the number to the left of the decimal point will quickly tell you the approximate titer of the serum. For instance, a titer of 2.4 is a dilution of at least 10^2 or 1 to 100; and since the titer is less than 10^3, the dilution cannot be greater than 1 to 1,000.

Another factor that must be considered is the type of serologic test employed. With certain virus diseases such as bluetongue, complement fixation is a broad spectrum test since any serotype of bluetongue virus will react with an antiserum produced by infection with any other serotype. If one wishes to identify the infecting serotype of BT virus, it is necessary to use the neutralization test since it is type specific. The complement fixation test is the obvious choice for confirming bluetongue infection. With other virus diseases such as foot-and-mouth or vesicular stomatitis, the complement fixation test is serotype specific and it is necessary to use all the different serotypes of the virus in the test.

The veterinarian should not hesitate to question the results, choice of test, or interpretation furnished by the laboratory. Only by frank and open exchange can some of these difficulties be worked out.

Application of Serologic Test to Specific Diseases

For each disease there are specific advantages and limitations to the use of serology. There are common factors related to the effect on antibody titers such as the ingestion of colostrum, vaccination, administration of antiserum, and infection with antigenically related viruses. These will be discussed in some detail as they pertain specifically to hog cholera as an example.

Hog Cholera (HC). Antibodies against HC may be detected using a fluorescent antibody neutralization test that is based on the ability of the serum to interfere with the infection of pig kidney cell cultures. Fourfold dilutions of the serum are prepared (1 to 4, 1 to 16, 1 to 64, 1 to 256, 1 to 1024, etc.) and mixed with a standard dose of HC virus. The virus-serum dilutions are placed on susceptible cells and the cultures are incubated overnight. The cell cultures are then stained with the anti-HC fluorescent antibody conjugate and examined for fluorescence. Detection of fluorescence is proof that free virus was available to infect the cells and the serum dilution lacked antibodies.

Within 14 to 21 days after infection with HC, surviving pigs developed titers of 1 to 4 (Log_{10}, 0.6) to 1 to 16 (Log_{10}, 1.2) and within another 2 or 3 weeks had titers of 1 to 64 to 1 to 1024 (1.8-3.0). Following vaccination with attenuated live HC virus, pigs had titers in the range of 1 to 16 to 1 to 256. If these vaccinated pigs were inoculated with virulent virus, the titers rose to 1 to 256 or higher. The titers following vaccination with live virus were found to persist in closed herds for 3 to 4 years. On the contrary, vaccination with killed (crystal violet) HC virus produced low titers of 1 to 4 in 3 of 12 pigs receiving one dose of vaccine. Some of these pigs became sick following inoculation with virulent virus.

The ingestion of colostrum by baby pigs from HC immune sows with high antibody titers caused the sucklings to develop serum titers as high or higher than the sows. Some of these passive antibody titers persisted for up to 6 months. The same persistence of passive antibody titers was found in pigs following the inoculation of HC antiserum in large doses of 1.0 ml per pound body weight.

The cross neutralizing titers against HC produced by infection with bovine virus diarrhea (BVD) have caused problems in interpreting the test results. Pigs are found naturally infected with BVD virus usually from close association with cattle. Only low HC titers of 1 to 4 to 1 to 16 were produced while the same pigs had BVD titers of 1 to 256 to 1 to 1024. It is often necessary to test serums against both viruses in order to make certain the low HC titers are cross reactions produced by BVD infection.

The detection of HC antibody titers of 1 to 64 or higher in a herd of pigs is cause for intensive investigation and epidemiologic study. A pregnant sow with a HC antibody titer may have been infected during pregnancy and could be carrying infected baby pigs in her uterus. If it is not possible to detect the virus, it is difficult to justify depopulation. However, tracing of pig movements to and from the herd may lead to the location of the active HC infection.

Other Swine Viruses, Pseudorabies, Transmissible Gastroenteritis, and Swine Enteroviruses. The swine viruses listed above are all cytopathogenic on the appropriate cell culture systems so neutralization tests may be performed on paired serums to check for increases in antibody titers. However, there are eight different serotypes of the enteroviruses so it is considerable work to test a set of serums unless the infecting serotype has been identified.

Infectious Bovine Rhinotracheitis (IBR). This is a cytopathogenic virus in bovine cell cultures and neutralization titers following infection range from 1 to 16 to 1 to 64. Paired serums are most productive since many cattle are vaccinated for IBR or have nursed dams with high antibody titers. Following vaccination, the titers may be quite low depending on the type of vaccine used. If the cattle were negative, i.e., <1 to 2, before vaccination, post vaccination titers may range from 1 to 2 to 1 to 8.

There is little value in collecting paired samples from aborting cattle. By the time the abortion occurs the cow has developed an IBR titer and a serum collection 3 to 4 weeks later may not have a significant increase (more than two dilutions).

Bovine Virus Diarrhea (BVD). Since some strains of BVD virus are cytopathogenic, laboratory strains have been developed for use in the neutralization test. Tremendous titers of 1 to 256 to 1 to 1,024 are found following infection and even passive antibody titers from the ingestion of colostrum will last for 7 to 8 months. Due to the many subclinical infections with BVD, paired serums are a necessity. Of course, the two or three animals in the herd suffering from mucosal disease are also infected with BVD virus but do not develop antibody titers. Instead, the animals become tolerant of the virus and high concentrations of virus are found in their blood and tissues. The BVD virus may be readily cultured from these cattle.

Parainfluenza-3 (PI-3) or Shipping Fever Virus. This virus is usually found associated with *Pasteurella spp.* but is undoubtedly a pathogen in its own right. A hemagglutination-inhibition test may be performed on paired serums. Titers following infection may range from 1 to 80 to 1 to 320. Due to the many strains of influenza virus, both human and animal, it is almost impossible to find cattle without low titers to PI-3.

Bluetongue. A complement-fixation or immunodiffusion test may be used to detect antibody responses to bluetongue infection. The complement-fixation titers recede quickly following clinical recovery, usually in less than 6 months, while the positive reactions detected by the immunodiffusion test may persist much longer.

Complement-fixation titers of 1 to 20 to 1 to 320 may be found following infection. Cattle may have bluetongue without having clinical signs of illness.

Leptospirosis. There are microscopic and plate agglutination tests for antibodies against leptospira. It is important to suggest the probable infecting serotype and submit paired samples. There are over 14 different leptospiral serotypes found in the U. S. and many normal animals have low titers of 1 to 100 to the common types of leptospira.

Equine Infectious Anemia (EIA).—The immunodiffusion (Coggins) test for EIA is a valuable diagnostic tool since it detects the quiescent carrier animal as well as the horse that is having recurrent attacks of febrile anemia. Since almost every horse infected with EIA develops a lifelong viremia, the horse that is positive to this test is an undesirable member of the equine community. Foals born to EIA infected horses will have a positive reaction to the test due to colostral antibody. However, these foals are not usually infected with EIA and will become negative unless they are infected. Neonatal infection of the foal with EIA is usually associated with infection of the mare during pregnancy.

Venezuelan, Eastern, and Western Equine Encephalitis. There are two serologic tests used for the encephalitis viruses, the hemagglutination-inhibition (HI) and the plaque reduction, neutralization test. The HI test is useful for paired serums since titers of 1 to 10 to 1 to 320 are found following infection. Unfortunately, some horses that are infected with either Eastern or Western virus develop strong cross reactions with Venezuelan. It is necessary to test these serums with the plaque reduction, neutralization test to determine whether these Venezuelan titers are specific. With this test the serums are tested at dilutions of 1 to 10 and 1 to 100. A 90 percent reduction of the plaque count of the virus by the 1 to 10 serum dilution is considered evidence that the horse was previously infected with Venezuelan encephalitis virus.

Equine Rhinopneumonitis and Equine Virus Arteritis.—Neutralization tests may be performed on paired serums for these equine disease since both viruses are cytopathogenic. However, by the time the abortion occurs, the mare has usually developed a titer to the virus.

Viscerotropic, Velogenic, Newcastle Disease. Although this virulent strain of Newcastle disease virus is named after its ability to produce hemorrhagic and necrotic lesions of the gastrointestinal tract, it may also be identified by its unique ability to produce red plaques on chick embryo cell cultures. Domestic velogenic strains produce clear plaques while vaccine strains do not produce plaques at all unless certain additives are included in the culture medium. The hemagglutination-inhibition test works particularly well with Newcastle disease virus. A random sampling of serums from a chicken flock may be tested for the purpose of determining the level of immunization following vaccination or for the presence of a significant percentage of high titers, 1 to 160 to 1 to 320, caused by infection with a velogenic strain of a Newcastle disease virus.

Conclusion

It is hoped that the information presented here will enable the veterinarian to develop a better working relationship with the laboratory and understand some of the problems involved in the interpretation of serologic tests. The extra effort to obtain paired samples and resolve the diagnostic problem of the stockman is certainly a practice builder, particularly so in this new era of agricultural combines where one organization may control the operations of many different farms. However, it must be remembered that most modern disease problems have a dual or even multiple etiology and the presence of an infectious agent may not be the only cause of the illness observed. Reliance on laboratory results alone without considering other facets of the problem may lead to failure. The assistance of the laboratory will be more effective when the veterinarian understands the inadequacies as well as the advantages of the confirmatory techniques employed.